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Delaying Training after Spinal Cord Injury Decreases Functional Recovery

by

Bryan Alexander Norrie

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

Department of Biomedical Engineering

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Abstract

The purpose of this study was to examine if delay between spinal cord injury (SCI) and rehabilitative motor training affects functional motor recovery. We studied a skilled motor task in which spinal cord injured rats traverse a horizontal ladder, focusing on accurate foot placement of the affected hind limb. Rats were trained to walk on the ladder without error (foot slips between the rungs), then treated with a T8 dorsal quadrant lesion to unilaterally sever the corticospinal and rubrospinal tracts. The animals displayed little to no permanent locomotor deficit on a flat surface (18-21 on the Basso-Bresnahan-Beattie (BBB) open field locomotor score), but exhibited a mean error of 64% in the affected limb when crossing the ladder. Performance did not improve over time if the animals were not exposed to the ladder for 3 months after injury. Animals trained immediately after injury (3 days) improved to 23.8% error after 3 weeks of training. Animals that sustained a 3-month delay period before training improved only to 51.9% error after 3 weeks of training, a reduction in improvement of 28.1% compared to the immediately trained group. Histological reconstruction of lesion extent was performed and this measure correlated well with the initial (pre-training) error and improvements made in stepping accuracy after training. Animals were then matched according to their lesion profile to verify that the behavioral differences observed were not due to differences in lesion profile between groups. Following this, a large discrepancy in eventual improvement was still evident, i.e., immediately trained animals improved 30% better than 3-month delay animals even though the extent of the spinal cord damage was the same. Modulation of reflexes (tactile and proprioceptive placing, and ground support response) was not correlated with functional improvements. Our results indicate that an optimal timeframe exists after SCI for maximum functional recovery via motor training.

To my parents, who taught me to love learning.

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Table of Contents

Chapter 1: Introduction

1.1. Introduction to Locomotor Training 1

1.2. Introduction to Spinal Cord Injury 2

1.3. Spinal Cord Injury and Locomotor Function 4

1.4. The Corticospinal Tract – Anatomy 5

1.5. The Rubrospinal Tract – Anatomy 7

1.6. CST and RST Functions and Interactions 7

1.7. RST Damage 10

1.8. CST Damage 11

1.9. Training and Rehabilitation 12

1.10. Training Humans vs. Training Animals 12

1.11. Locomotor Assistance – Electrical and Mechanical 15

1.12. Mechanisms of Recovery 15

1.13. Fast Plasticity – Long Term Potentiation 16

1.14. Slow Plasticity – Synaptogenesis and Sprouting 17

1.15. Sprouting 18

1.16. Plasticity in Injury and Recovery 20

1.17. Training and Functional Recovery – Time Course 21

1.18. Task-Specific Improvement without Training 22

1.19. Training Paradigm – Horizontal Ladder 23

1.20. References 27

Chapter 2: Impairment of Functional Recovery Due to a Time Delay between Spinal Cord Injury and Motor Training

2.1. Introduction 37

2.2. Methods 40

2.2.1. Training 40
2.2.2. Surgery 41
2.2.3. Delay Protocol 41
2.2.4. Histology 42
2.2.5. Reconstruction of Lesion Site 42
2.2.6. Reflexes and Muscle Stiffness 44
2.2.6.1. Tactile Placing Response 44
2.2.6.2. Proprioceptive Placing Response 45
2.2.6.3. Ground Support Response 45
2.2.6.4. Muscle Stiffness 45
2.2.7. Statistics 46

2.3. Results 46

2.3.1. Lack of spontaneous recovery in accurate stepping 46
2.3.2. Effect of delay on training response 47
2.3.3. Comparison of accurate stepping and lesion size 48
2.3.4. Animals further matched by histological profiles 50
2.3.5 Comparison of stepping strategy 50
2.3.6. Time course of recovery of descending and segmental reflexes 51
2.3.6.1. Ground support response 51
2.3.6.2. Tactile and proprioceptive placing response 52
2.3.6.3. Passive Muscle Stiffness 54

2.4. Discussion 54

2.4.1. Comparison between groups: initial error 55
2.4.2. Comparison between groups: % improvement 56
2.4.3. Variability in lesion reconstruction 57
2.4.4. Reflex results 59
2.4.5. Mechanisms of training-induced recovery 59
2.4.6. Physiological effects of delay 61
2.4.7. Functional Implications 62

2.5. References 74

Chapter 3: Development of Electrophysiological Methods in the Waking Animal to Assess Corticospinal Plasticity

3.1. Introduction 78

3.2. Methods 80

3.2.1. Implantation of cortical stimulating screws and EMG electrodes 80
3.2.2. Recording of cortically evoked responses in the waking animal 81

3.3. Results 82

3.4. Conclusions 83

3.5. References 90

Chapter 4: General Conclusion

4.1. General conclusion 91

4.2. Spared CST/RST fibers 92

4.3. Accessory pathways 93

4.4. Propriospinal neurons 94

4.5. Corticorubral system 95

4.6. Training and synaptic remodeling 95

4.7. Effects of a time delay before training 97

4.8 Future directions 98

4.9. References 99

List of Tables

Chapter 2: Impairment of Functional Recovery Due to a Time Delay between Spinal Cord Injury and Motor Training

Table 2.1. Histological and Performance Profile of Lesioned Animals 63

Table 2.2. Group Averages for Matched Subset 64

List of Figures

Chapter 1: General Introduction

Figure 1-1. The Human Corticospinal Pathways 25

Figure 1-2. The Red Nucleus and Rubrospinal Tract 26

Chapter 2: Impairment of Functional Recovery Due to a Time Delay between Spinal Cord Injury and Motor Training

Figure 2-1. Demonstration of horizontal ladder and stepping error 65

Figure 2-2. Spinal cord histology 66

Figure 2-3. Comparison of initial error between groups 67

Figure 2-4. Comparison of error throughout training in both groups 68

Figure 2-5. Lesion vs. initial error for total group 69

Figure 2-6. Lesion vs. improvement for total group 70

Figure 2-7. Lesion vs. initial error and improvement for matched group 71

Figure 2-8. Ground support response in immediate and delay trained animals 72

Figure 2-9. Proprioceptive placing response in immediate and delay trained animals 73

Chapter 3: Development of Electrophysiological Methods in the Awake Animal to Assess Corticospinal Plasticity

Figure 3-1. Stimulation specifically evokes activity in tibialis anterior 82

Figure 3-2. Stimulus-response curve 83

Figure 3-3. Evoked response before and after spinal injury 84

List of Abbreviations

APV, transcription factor activator protein 5

BBB score, Basso-Bresnahan-Beattie open field locomotor score

BDNF, brain-derived neurotrophic factor

cCST, contralateral corticospinal tract

CNS, central nervous system

CPG, central pattern generator

CST, corticospinal tract

EMG, electromyogram

EPSP, excitatory post-synaptic potential

FES, functional electrical stimulation

GABA, gamma-amino butyric acid

GAP-43, growth-associated protein 43

iCST, ipsilateral corticospinal tract

iRST, ipsilateral rubrospinal tract

LTP, long term potentiation

MEP, motor evoked potential

MG, medial gastrocnemius

mRNA, messenger ribonucleic acid

NMDA, N-methyl-D-aspartate

NT-3, neurotrophin-3

PBST, posterior biceps semitendinosus

PVC, polyvinyl chloride

RST, rubrospinal tract

SCI, spinal cord injury

T8, thoracic segment 8

TA, tibialis anterior

TMS, transcranial magnetic stimulation

TrkB, tyrosine receptor kinase B

TrkC, tyrosine receptor kinase C

VL, vastus lateralis

<Chapter 1>

General Introduction

1.1 Introduction to Locomotor Training

Impressive improvements in functional motor recovery result from intensive daily motor training (Muir and Steeves, 1997; Wernig et al., 1995; de Leon et al., 1998; Barbeau and Rossignol, 1987), and the effects of training are retained for some time after the training sessions conclude (De Leon et al., 1999; Wernig et al., 1998). Based on clinical studies, it appears that timing of the onset of training may have an impact on the extent of functional motor recovery achieved through training. However, due to the constraints of the clinical studies (lack of experimental control resulting in extensive lesion variability and variability in delay period before training begins), large gaps remain in our knowledge of this subject. In order to systematically explore the effects of a delay period after spinal cord injury before the onset of training, an animal model must be developed. In this manuscript, we present the design and implementation of such a model, and thereby demonstrate that, in our model, a delay in the onset of locomotor training following spinal cord injury does indeed produce a reduction in the functional benefits achieved by training.

1.2 Introduction to Spinal Cord Injury

Traumatic spinal cord injuries occur at a rate of approximately 10,000 per year in the United States, primarily due to motor vehicle accidents, violence, falls, and recreational activities. The incidence rate is greatest in young people (15-25 years), and four times as prevalent in males as in females. In addition to traumatic spinal cord injury, non-traumatic spinal cord injuries such as neurodegeneration, cancer, polio, and autoimmune disorders (Kirshblum et al., 2002) are also major causes of similar pathologies, and are as much as four times as prevalent as traumatic injuries (McDonald et al., 2002).

Following the initial insult, which typically compresses or severs the nervous tissue of the spinal cord by displacement of bone and other spinal tissues, the spinal cord begins to swell. Damage to blood vessels produces ischemia, and this is exacerbated by both the cord swelling and the onset of neurogenic shock which produces systemic hypotension (Sekhon and Fehlings, 2001). Ischemia may also be increased by the occurrence of thrombosis and hemorrhage. In addition to ischemia, release of toxic chemicals (e.g., free radicals) from disrupted cell membranes and altered local electrolyte concentrations extend the damage done by mechanical forces. Glutamate released from damaged neurons causes excitotoxicity in proximal neurons, seemingly by producing sustained elevation in intracellular calcium which activates apoptotic pathways (Agrawal and Fehlings, 1997; Liu et al., 1999).

Axotomy alone does not immediately kill most neurons; instead the cell typically eliminates the remaining portion of the axon proximal to the injury site (Tetzlaff et al., 1994). This occurs in part because regeneration of axons in the adult central nervous

system is very rare due to a number of neurite growth inhibitors expressed in the brain and spinal cord (Tatagiba et al., 1997). NI-35/250 (Cadelli and Schwab, 1991), myelin associated glycoprotein (McKerracher et al., 1994), and collapsin (Luo et al., 1993) are members of this growing family that have been studied in detail. These molecules have the effect of repelling axonal growth cones, or even causing complete retraction, whereby the growth cone disappears and the extending axon disintegrates (Li et al., 1996). These retracting growth cones are known as retraction bulbs, which appear within a day after spinal cord injury, and many persist for at least a month, although continuous application of ex[illegible]ous neurotrophins can reduce this degenerative effect, presumably encouraging t[illegible]rites to form synapses locally (Sayer et al., 2002). However, although many of the supraspinal neurons remain alive after axonal severance (Kwon et al., 2002), and may even undergo plastic changes in their connection patterns (discussed in detail later), they can no longer directly exert any effect on activity above and below the lesion site.

Demyelination is another important feature of spinal cord injury. Within a week after trauma to the spinal cord, a process known as Wallerian degeneration begins, leading to eventual apoptosis of at least some myelin-forming oligodendrocytes (Warden et al., 2001). Loss of myelin in otherwise intact axons can cause slowing and dispersion of axonal conduction, creating an effective blockade of conduction (Raynor et al., 1995; Brouwer et al., 1992), rendering these connections useless, despite the integrity of both the cell body and descending axons.

1.3 Spinal Cord Injury and Locomotor Function

With the loss of descending input comes loss of function. This review will focus on locomotor and skilled movements, but disruption of bladder function, damage to the autonomic nervous system, sensory loss, and sexual dysfunction all commonly result from spinal cord injury (McKinley et al., 2002).

In animal studies, fictive locomotor behavior in hindlimb muscles can occur after the elimination of descending and afferent inputs. This is because the spinal cord contains a central pattern generator, a system of interneurons and motor neurons that is capable of generating rhythmic, coordinated activity in locomotor muscles with little or no supraspinal input (McCrea, 2001). However, in humans, the central pattern generator is more dependent upon descending activation from supraspinal connections (Butt et al., 2002), particularly the corticospinal or pyramidal tract, which descends from the primary motor ($1/3^{rd}$), somatosensory ($1/3^{rd}$) and premotor ($1/3^{rd}$) cortices (Vilis, 2002) to most levels of the spinal cord (Leenen et al., 1985c). In humans, severance of the corticospinal tract alone produces an irreversible loss of the ability to walk (Nathan, 1994), whereas rats can completely recover overground locomotor abilities following pyramidal tract section (Castro, 1972; Muir and Whishaw, 1999). Moreover, while reflex activity can produce some movement in patients with complete spinal cord injuries, it is not sufficient to evoke an accurate locomotor pattern capable of weight support (Dietz et al., 1994; Wernig et al., 1995).

Because of this fundamental difference between the organization of the human and rat (and cat) locomotor pathways, direct comparison of the supraspinal control of the two systems isn't feasible. It is likely that the increased element of supraspinal control

required in human locomotion is due to its bipedal nature – balance and weight support are a greater concern for bipeds than for quadrupeds. Further, many functions served by developmentally older tracts (e.g., rubrospinal) in animals appear to be taken over by the corticospinal tract in humans (see below). Thus, in order to utilize animal models to broaden our understanding of human motor control and spinal cord injury, we must exploit some of the similarities between the systems, and be aware of the important differences.

1.4 The Corticospinal Tract - Anatomy

The corticospinal tracts (CST), discussed briefly above, and the rubrospinal tract, which extends from the red nucleus of the midbrain to many levels of the spinal cord, are well-preserved in rats, cats and, to a lesser extent, in man. While the organization of the descending tracts varies somewhat between species, the distribution and function of the tracts have some basic similarities. After a brief discussion of the comparative anatomy and neurophysiology of these motor systems, we will proceed with exploring the consequences of their damage and the prospects for locomotor recovery.

In the primary motor cortex, large pyramidal cell bodies exist in layer V. The axons of these cells descend through the internal capsule and descend caudally to the medullary pyramids (it is due to the passage near the medullary pyramids, not the shape of the cells, that the tract is sometimes called the 'pyramidal' tract, which refers to the CST and the corticobulbar tract). At the pyramids, this large tract containing approximately one million axons (Leenen et al., 1985) splits into two separate tracts (see **Figure 1**). In humans, one tract forms the lateral CST, which contains approximately 75-

90% of the total CST axons, and decussates at the pyramids and descends in the contralateral dorsolateral column. The second tract, the ventral CST, does not cross the midline, but descends via the ventral column. The ventral fibers terminate bilaterally in the ventromedial cell column and the intermediate zone, primarily synapsing onto interneurons near the motor neurons of the axial muscles. The lateral CST projects to motor nuclei in the lateral grey matter (laminae IX) and to interneurons of the intermediate zone (Casale et al., 1988). Approximately 5-6% of the fibers are unmyelinated both in humans and in rats (Meier, 1976).

The rat corticospinal tract comprises approximately 100,000 axons, with a cross-sectional area of about $0.3mm^2$, see also (Leenen et al., 1985). However, a major difference between the rat and human CST is that there are very few fibers in the rat that course through the lateral funiculus. While a very small portion does descend in the ventral funiculus, 90-95% of the rat CST decussates and travels along the ventromedial aspect of the dorsal funiculus. The ipsilateral (uncrossed), ventral CST is similar in rats and humans, although in rats the fibers descend to the lumbar portion of the spinal cord (Joosten and Bar, 1999), and synapse into the grey matter at all levels (Brosamle and Schwab, 1997). Interestingly, in marked contrast to the topographic organization observed in the sensorimotor cortex (Neafsey et al., 1986), an anterograde labeling study by Jeffery demonstrated that the CST at the cervical level displays no topographic organization (Jeffery and Fitzgerald, 1999).

1.5 The Rubrospinal Tract - Anatomy

The rubrospinal tract (RST) originates in the magnocellular portion of the red nucleus (see **Figure 2**). It decussates in the midbrain and courses along the lateral aspect of the brainstem, then more caudally in the contralateral dorsolateral column, projecting into the dorsolateral spinal grey matter. While it has been shown that the RST plays a role in accurate movements of distal limb muscles in cats and monkeys (i.e. reaching and grasping motions, (Jarratt and Hyland, 1999), it is questionable whether the RST is important in the control of human movement, as the tract is small and very few fibers project into the spinal cord (Nathan and Smith, 1982). However, the presence of a well-conserved RST in animals that do not make skilled movements of the distal limbs (Muir and Whishaw, 2000) suggests additional functions for this tract (discussed below). The caudal magnocellular portion of the red nucleus, from which the RST originates, is comparatively small in humans, and the majority of projections from the red nucleus are from the parvocellular zone to the reticular formation and the olivary nuclei.

There are no direct connections from the RST onto alpha motoneurons in the rat (Kuchler et al., 2002). Somatotopic arrangement seems to take place, with cells from the ventrolateral part of the nucleus synapsing onto lumbar regions (Kostyuk and Pilyavsky, 1969).

1.6 CST and RST Functions and Interactions

The roles of the CST and RST are fairly similar in both rats and cats, with many functions overlapping, or perhaps more accurately, one system compensating for the other following injury, allowing sparing of function (Leonard and Goldberger, 1987). The

red nucleus receives many inputs from the motor cortex, presenting the possibility that the cortico-rubrospinal tract acts as an accessory to the CST. The tracts share a function in mediating reaching and grasping motions in the forelimbs. This contrasts with humans and other primates, in which the cortex alone is responsible for intricate hand and finger movements (Lawrence and Hopkins, 1976), although the motor cortex does seem to play a larger role in rats as well; but even with both the CST and RST transected, some grasping function remains (Whishaw et al., 1998). Indeed, the similarities in function have led some researchers to comment upon the parallel, possibly competitive role of the two pathways. Two ablation studies particularly highlight the sometimes interchangeable nature of these tracts. In the first study, after CST transection, IN-1 antibody was applied (to block the neurite growth inhibiting effect of NI-35/250), leading the RST to reorganize, growing functional synapses into the area left vacant by the severed CST fibers (Raineteau et al., 2002). Similarly, while ablation of the RST alone is insufficient to induce collateral sprouting of the CST, when neurotrophin-3 is added as well, there is an increased density of innervation by the CST into the superficial dorsal horn where RST endings are normally found (Jeffery and Fitzgerald, 2001). Moreover, in neonatal rats, if the CST is transected, cells from the motor cortex will increase their connections to the red nuclei (Z'Graggen et al., 2000), with impressive bilateral overlap not usually seen (Murakami et al., 1993) and there is evidence that the red nucleus may act to alter the functions of spared descending fibers following injury (Fanardjian et al., 2000).

During locomotion in rats and cats, the CST and RST seem to be more involved in responses to the surrounding environment of the animal, with heavy involvement in changes in gait, accurate stepping and obstacle avoidance. Using extracellular recording,

Armstrong demonstrated that the motor cortex is more active when cats walk across a horizontal ladder (requiring accurate foot placement) than simple overground or treadmill walking (Armstrong, 1986). Animals negotiating their way over uneven terrain such as crushed rock (Keifer and Kalil, 1991), or stepping over obstacles placed on a treadmill (Beloozerova and Sirota, 1993) require intact motor cortex for accurate performance of the task. In the cat, Drew also demonstrated activity in motor cortex cells that corresponded to voluntary gait modifications made by cats to step over obstacles placed on a treadmill belt (Drew, 1988). Pyramidal tract neurons have also been implicated in both change of limb trajectory, and postural anticipation of this change. However, corticoreticular neurons are more active prior to the actual change, indicating that the corticoreticular system is more important for postural preparation for planned deviations from the motor pattern (Kably and Drew, 1998). While disruptive in complex tasks such as those mentioned above, CST lesions have virtually no effect on simple overground locomotion in rats. Castro commented more than 30 years ago that due to the subtle nature of the motor deficits produced by pyramidal tract transection, appropriate methods are required to study behavioral changes and subsequent recovery (Castro, 1972). Indeed, in a detailed kinematic and ground reaction force study, Muir reiterated that rats can completely recover normal overground walking behavior (appropriate stepping, coordinated electromyogram (EMG) activity, kinematic pattern, etc.) following bilateral pyramidal tract section (Muir and Whishaw, 1999).

1.7 RST Damage

Studies of RST transection alone aren't common, as most such studies involve lesions of the dorsal columns and dorsolateral funiculi, which produce combined deficits in the RST and CST. However, in a simple overground or treadmill walking task, animals can also compensate for these injuries with minimal lasting deficits. In cats, an incomplete bilateral lesion of the dorsolateral funiculi and dorsal columns produced little lasting deficit, and even complete dorsolateral lesions produced only modest changes in EMG activity and kinematic patterns (Jiang and Drew, 1996). Axotomy of the RST does not generally affect the termination sites of red nucleus cells onto other supraspinal centers (Wang et al., 2000) allowing the possibility that the cells continue to exert some effect indirectly.

Lesions of the red nucleus itself have sometimes led to overestimates of the functional deficits produced, as electrolytic lesions can affect fibers of passage nearby. Whishaw demonstrated that using microinjection of ibotenic or quinolinic acid, which damage cell bodies only, far less behavioral deficits (such as postural instability, problems grasping objects) were apparent then if standard anodal electrolytic lesions were performed (Whishaw et al., 1992). In addition to alterations in kinematic pattern, the red nucleus may have a more general role in locomotor control. Kainic acid lesions of the red nucleus produce impairment of accuracy in reaching tasks in rats, as well as creating a delay in the initiation of such movements. While the accuracy can be recovered with training, the elevated latency remains (Lorincz and Fabre-Thorpe, 1997). Similarly, loss of the ipsilateral RST causes loss of conditioned forelimb responses (but not unconditioned responses) in a way that CST transection does not, leading Voneida to

speculate that the RST may act as a trigger for the expression of learned motor responses (Voneida, 1999). Whishaw proposed that the red nucleus may provide a tonus to the limb, giving distal muscles a framework from which to perform accurate movements such as grasping and avoiding obstacles (Whishaw and Gorny, 1996).

1.8 CST Damage

Damage to the CST produces errors in performance of complex motor tasks such as accurate placing or skilled reaching (Whishaw and Metz, 2002). A temporary increase in extensor activity occurs soon after CST lesion; it appears that loss of inhibitory interneuron input to the extensor motor neuron pool mediate this extensor activity, as the EMG activity of flexors remains essentially unchanged (Eidelberg and Yu, 1981). In addition, CST damage causes deficits in the conditioning of reflexes (Chen et al., 2002b). Using retrograde labeling techniques, the damage to these descending fibers can be quantified (by performing counts of labeled cells) and compared with functional deficits (Midha et al., 1987).

While damage to the CST and RST cause functional deficits, these deficits are not necessarily permanent, particularly locomotor ones. Functional recovery often occurs, spontaneously or in response to motor training and other therapies. The next portion of this review will focus on strategies for improving functional motor recovery following spinal cord injury, followed by a review of the mechanisms underlying functional recovery, especially synaptic plasticity.

1.9 Training and Rehabilitation

Recovery of locomotor functions compromised by spinal cord injury can be achieved by locomotor training. This requires that the spinal cord receives specific sensory input via performance of the appropriate motor task, so that training techniques that assist the accurate performance of desired movements will help to develop the spinal circuitry for improved performance (Behrman and Harkema, 2000). All of the techniques described include numerous repetitions, over time, of the intended movement (i.e. stepping) or the closest approximation possible of the intended movement, in order to encourage the nervous system to learn and remember the motor pattern.

Treadmill training has become common practice in many rehabilitation regimens for recovery of locomotor skills after spinal cord injury (Wernig et al., 2000) and stroke (Hesse et al., 2001). While the benefits are limited to patients with incomplete injuries (Dietz et al., 1998), improvements in walking speed and reflex function can be made in a single bout of training (Trimble et al., 2001). Cats are routinely trained to walk on treadmills, and a few studies have shown that treadmill training also benefits rats after spinal cord injury (Thota et al., 2001; Fouad et al., 2000).

1.10 Training Humans vs. Training Animals

The loss of descending fibers decreases the efficacy of intraspinal and afferent synaptic activation of locomotor cells (the central pattern generator (CPG) and motoneurons). Training animals to walk on a treadmill, even in the absence of any remaining descending projections, can improve motor performance (Edgerton et al., 2001) with significant limitations. The tract extending along the ventrolateral funiculus of

the spinal cord from the medullary reticular formation is implicated in general locomotor activity such as maintained gait patterns. It appears to exert inhibitory influence on alpha- and gamma-motoneurons and interneurons of reflex pathways (Takakusaki et al., 2001). Preservation of the reticulospinal tract (Schucht et al., 2002) after varied spinal cord injuries in rats was the strongest correlate with a high BBB score (a 21-point scale for simple overground locomotion in rats; (Basso et al., 1995). The vestibulospinal tract is another major descending system, mediating aspects of balance and posture during locomotion (Nathan et al., 1996). Brustein and Rossignol performed lesions of the ventrolateral funiculus in cats, and discovered that extreme damage to the vestibulospinal and reticulospinal tracts could be compensated for, to a large extent, by remaining dorsolateral pathways (Brustein and Rossignol, 1998).

It has been demonstrated that increases in locomotor activity (exercise) can produce increased levels of the neurotrophins brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) in both the muscles and spinal cords of rats (Gomez-Pinilla et al., 2001). These neurotrophins are known initiators of axonal growth and regeneration, and thus may contribute to functional and anatomical plasticity (Schnell et al., 1994). However, in order to translate this increase into improvement of certain functions, those functions must be practiced, reinforcing the proper connections in an activity-dependent manner (Muir and Steeves, 1997). For instance, when training spinal cats, de Leon demonstrated that cats could be trained to stand in place, or to walk with weight support, but no overlap was observed in the standing abilities of the cats that were trained to walk, or the walking abilities of the cats that were trained to stand (de Leon et al., 1998a; de Leon et al., 1998b). In this respect, the benefits of training are fairly activity-specific.

One promising aspect is that the improvements in performance are maintained long after the training period ends (de Leon et al., 1999), suggesting lasting effects of rehabilitation regimens. This is corroborated by human studies, in which the benefits remain several months after termination of the training program (Wernig et al., 1998; Wirz et al., 2001).

Cats with complete spinal injuries can be trained to produce full weight-bearing stepping on a treadmill (Edgerton et al., 1992; Lovely et al., 1990), or simple standing (De Leon et al., 1998a), with EMG patterns similar to those of an intact animal. No descending input remains in these animals; the locomotor pattern is evoked simply by sensory inputs such as the moving treadmill belt causing movements in the hind limbs. Additional perineal stimulation is often required to increase the amount of overall afferent input to the cord. Adult spinal rats can also be trained to step, although with more difficulty, but the afferent control of stepping is quite similar in rats and cats (Pearson, 2001). This is in contrast to humans, where complete transection of descending inputs entirely disrupts walking (Nathan, 1994).

Because cats and rats can learn to walk with little or no descending control, study of normal walking recovery in these animals is an incomplete model for human application – descending control is required in adult human locomotion. In order to compare human and animal training paradigms more directly, a training model involving descending control in animals is required. As discussed previously, more complex motor tasks require descending control in rats and cats, as has been demonstrated on uneven and curved surfaces and horizontal ladders for nearly 60 years (Liddell 1944). While the functions served are not homologous, the requirement of descending control is, providing

the possibility to train animals at complicated tasks after injury, and then study both the behavioral results and the underlying mechanisms of recovery

1.11 Locomotor Assistance – Mechanical and Electrical

Before proceeding to the underlying mechanisms of training-induced learning and recovery, some discussion of novel training mechanisms is warranted. Because it has been established that the main effector of training to alter spinal and supraspinal connections is afferent input (Pearson, 2001; Helgren and Goldberger, 1993), some new therapies have sought to exploit this by providing appropriate sensory information in spite of the inability of the motor system to perform the prescribed movements accurately on its own. Both in humans (Colombo et al., 2000) and in rats (De Leon et al., 2002), robotic gait trainers have been developed in order to assist the limbs to make appropriate movements. Manual assistance has also been used, but the robots have been shown to be more labor-efficient than, and at least as effective as, manual therapy in stroke patients (Werner et al., 2002). Similarly, muscular movements can be strengthened by direct electrical stimulation (known as functional electrical stimulation or FES), enabling them to perform accurate movements even when synaptic drive is wanting (Popovic et al., 2001).

1.12 Mechanisms of Recovery

Plasticity of synaptic circuits underlies all learning, as well as recovery of functions lost due to injury. Indeed, the morphological changes following CNS injury are typically quite similar to changes that take place during learning (Ivanco and Greenough,

2000). Aspects of this plasticity may take place in the timeframe of seconds to minutes, or on the order of days, weeks or even months (discussed below). The mechanisms mediating neural plasticity are diverse and complex. A large amount of what is known comes from studies on reduced preparations of simple nervous systems (Antonov et al., 2001), although a significant amount of work has been done on the mammalian hippocampus, which is believed to be a major site of memory acquisition (Sorra and Harris, 2000). This section will first explore some of the fundamental concepts of neural plasticity, then delve into more detail as the plasticity relates to the cells and systems involved in motor control, specifically as they pertain to recovery after injury.

1.13 Fast Plasticity – LTP

While an intensive review of the subject is beyond the scope of this paper and can be found in (Bailey et al., 2000), a brief description of the important features of the phenomenon of long term potentiation (LTP) is useful for our purposes. Hebbian plasticity refers to changes in the connective properties of a synapse due to coincident activation of the pre- and post-synatic neurons, although other neuromodulatory inputs may also be involved. The basis for LTP is appropriate activation of the post-synaptic cell, usually involving coincident depolarization with the pre-synaptic neuron (alleviating the magnesium block of NMDA-type glutamate receptors) and activation of the NMDA receptors, allowing calcium influx. This leads to many downstream events, some at the cell membrane (altering the synaptic and electrical properties through short term events such as phosphorylation cascades), some involving transcription of mRNA and protein expression, producing long-term changes in cellular properties (such as elevated

expression of neurotransmitter receptors, or sprouting of axonal and dendritic arbors). Various forms of LTP have been discovered, with differences based upon the type of cell and types of neurotransmitters, receptors and neuromodulators involved, but the basic tenets of coincident activation remain.

Afferent stimulation can increase the efficacy of synaptic connections in cultured cells, an effect that is reversed by the addition of transcription factor activator protein 5 (APV), an NMDA channel blocker. Further, lasting elimination of synaptic contacts occurs if the NMDA blockade is maintained for prolonged periods, indicating that the activation plays a role not only in increasing efficacy, but also in maintaining it (Nelson et al., 1990). Clinical studies have shown that use-dependent plasticity can occur within minutes, and can be reduced by dextromethorphan (an NMDA receptor blocker) and lorazepam (a type-A GABA positive allosteric modulator), suggesting the involvement in these neurotransmitters in at least some forms of cortical plasticity (Butefisch et al., 2000). A positron emission topography study also identified a potential role for the type-A GABA receptor in fast plastic changes (Capaday et al., 2000). While neurotrophins have typically been thought of as agents for neuronal survival and the outgrowth of neurites (discussed in the next section), BDNF can cause phosphorylation of NMDA receptors, increasing calcium conductance and thereby modulating synaptic activity on a fast timeframe (Slack and Thompson, 2002).

1.14 Slow Plasticity – Synaptogenesis and Sprouting

Temporary changes in synaptic efficacy induced by transient alterations of ionic conductance, receptor availability and neurotransmitter release can become more stable

following transcriptional processes. These include changes in neurotransmitter and neurotrophin release, expression and turnover of receptors, and structural changes in the neurons themselves. Formation of axonal arbors and dendrites can occur, as well as changes in synapse number and stability.

1.15 Sprouting

Sprouting is the most visible plastic change to occur, as with an appropriate tracing technique one can visualize the sites of neurite outgrowth and synaptogenesis (Fouad et al., 2001b). Sprouting is limited under normal conditions in order to prevent excessive plasticity, which could otherwise lead to loss of important connections or formation of aberrant ones like those implicated in chronic neuropathic pain and autonomic dysreflexia (Krenz et al., 1999). Generally outgrowing neurites follow a neurotrophin gradient, making target proximity a significant factor (there are intermediate targets during development to assist long axons to reach their destinations), and while poorly understood, topographical organization is often maintained from brain to spinal cord (Goldberger and Murray, 1982).

Sprouting and even regeneration following injury to the spinal cord during development is commonplace in mammals (Hase et al., 2002; Kuang and Kalil, 1990; Kalil and Reh, 1982). A number of strategies have therefore been exploited to try to replicate some of the conditions of the developing cord in order to improve functional plasticity in adults. These include application of neurotrophic factors (Rabchevsky et al., 2000), neutralization of growth-inhibitory molecules (Thallmair et al., 1998), and

providing a permissive environment as a 'bridge' across a lesion site (Li et al., 1998), among others.

Following injury, sprouting of collateral fibers takes place both in damaged and undamaged neurons, and while sprouting occurs spontaneously (without sensorimotor training), it can be potentiated by relevant activity (Raineteau and Schwab, 2001). A number of biochemical changes related to plasticity take place in the injured spinal cord. Following traumatic spinal cord injury, tyrosine receptor kinase C (trkC) receptor (receptor for NT-3) expression increases on descending axons (Frisen et al., 1993). Growth-associated protein 43 (GAP-43), an indicator of neuronal growth and plasticity, is also up regulated after spinal cord injury (Curtis et al., 1993). Axonal degeneration produces increases in neurotrophins and cell adhesion molecules in proximal glial cells (Jones et al., 1999). Axonal sprouting can be observed in severed corticospinal tract fibers beginning approximately 3 weeks after injury, and persists for at least 3 months, and reticulospinal fibers sprout with a slower onset (Hill et al., 2001). However, regeneration of severed fibers is very rare and limited in the adult mammalian central nervous system (CNS). By applying exogenous NT-3 and BDNF, Novikova prevented retraction of severed axons, but these would not grow into a peripheral nerve graft, let alone the growth-inhibiting CNS (Novikova et al., 2002). Successful axonal regeneration has been demonstrated into fetal tissue transplants (Coumans et al., 2001), but remains elusive in the adult CNS.

1.16 Plasticity in Injury and Recovery

Sensory input is the basis of training-related improvements in sprouting. While coincident activation of motor systems helps specify task-relatedness, activation of afferent fibers drives changes in connectivity, as demonstrated by the ability for plastic changes produced by 'passive' training (Carel et al., 2000). Simple exercise (running on a running wheel) evokes increases in BDNF and its receptor tyrosine kinase receptor B (trkB), synapsin I and GAP-43 in both spinal cord and muscle of rats following spinal cord injury, and paralysis caused by injection of botulinum toxin caused a drop off, suggesting that basal levels of these factors are contingent upon basal levels of motor activity (Gomez-Pinilla et al., 2002). Even simple environmental enrichment of the cages animals are housed in improves motor function and dendrite growth (Biernaskie and Corbett, 2001). Despite this, more pronounced effects are observed when animals are trained at particularly challenging tasks. Rats trained to walk across a thin, rotating pole (rota-rod) displayed greater synaptogenesis in motor nuclei of the thalamus than rats that were treadmill-trained (Ding et al., 2002). Similarly, acrobatic training led to an increase in synapse formation in layer V of the motor cortex, which was further enhanced by ischemic injury to part of the cortex (Jones et al., 1999). Rats trained at grasping motions after motor cortex ischemia displayed increased dendritic plasticity, and remarkably, showed greater plasticity and improvement in motor performance on the unaffected side than did control animals trained after a sham lesion (Bury and Jones, 2002). In the lamprey, 'physiologically relevant' stimulation causes alterations in glutaminergic and glycinergic connections, contributing to plasticity in locomotor networks (Parker and Grillner, 2000). Segmental excitatory post-synaptic potentials (EPSPs) are generally

enhanced following spinal cord injury; however, these changes are superimposed upon motoneuron hyperpolarization, so the net change in reflex activation is somewhat variable (Mendell, 1988).

1.17 Training and Functional Recovery – Time-Course

Injury to the nervous system creates an environment of heightened sensitivity to plastic changes (Garraghty et al., 1991), which may occur 'spontaneously' or in response to therapeutic intervention. Recently, the goal of post-injury therapies has shifted from working on compensatory strategies to training for the facilitation of reorganization of spared systems in order to maximize functional restoration (Field-Fote, 2000). While there is some evidence that early use of an affected limb after injury to the sensorimotor cortex actually increases the injury (Kozlowski et al., 1996), this is believed to be a local effect, presumably due to circulatory and metabolic constraints upon the cells at the site of the injury, as the same researchers demonstrated that early use did not enhance a non-cortical injury to the motor system, but that forced disuse did reduce functional recovery in this case (Bland et al., 2001). Furthermore, chronic disuse can lead to decreased levels of extracellular glutamate in the motor cortex (Bland et al., 1999), as well as plastic changes reducing the efficacy of transmission at the neuromuscular junction (Fahim, 1989). Monkeys subjected to section of the dorsal column of the spinal cord did not recover use of hand movements if the affected hand was placed in a cast at the time of injury (Asanuma et al., 1986). Conversely, Bregman found that by delaying experimental bridging of a spinal cord injury by transplantation was more effective several weeks later

than immediately after injury, although it is likely that reduced inflammation and immune presence contributed to better tolerance of the graft (Bregman et al., 2002).

Because the organization of the motor cortex is not static, but continuously modulated in a sensory-dependent manner (Chen et al., 2002a), context-dependent use after injury should act to maintain representation of tasks and muscle groups that are compromised by the injury. Because the properties of neural connectivity are continuously being shaped by use-related experience (Dorris et al., 2000), it stands to reason that the sooner one restores that experience, or the best possible approximation, the smaller the loss of original function should be. In addition to preservation of task-related connections, the motor activity and afferent information contributes to plastic changes in remaining neural pathways (Harkema, 2001; Raineteau and Schwab, 2001). Motor training produces rapid plastic changes (Classen et al., 1998), which are solidified by repetition into more stable changes in the connectivity of spared pathways that improve task-related efficacy (de Leon et al., 2001).

1.18 Task-Specific Improvement without Training

Although task-specific training is very important for improving recovery of function after injury, therapies that enhance general CNS plasticity can also have benefits in restoration of function. Following dorsal column lesions, rats lose the ability to accurately walk on a horizontal ladder, and without further exposure to the ladder, they make little or no improvement over extended periods of time (see Chapter 2). However, application of IN-1 antibody to eliminate the growth-inhibiting molecule NI-35/250 was effective in promoting spontaneous (no training) improvement in a number of complex

motor tasks, (including a 20% improvement for both hind feet on a horizontal ladder) (Fouad et al., 2001a). It is likely that other interventions, such as addition of neurotrophins, would have a similar impact. These therapies induce plastic changes in neural pathways, and although the plasticity may not be specific to the tasks being studied, if there is enough random remodeling of the system due to a vast increase in the number of connections made by existing neurons, some of the changes are bound to restore lost functions, or strengthen the pathways that mediate those functions. However, even if these plasticity-promoting therapies are advanced to the clinical level, concurrent training will still be necessary to maximize functional recovery.

1.19 Training Paradigm – Horizontal Ladder

In order to systematically study the effects of delaying training, one must use a model in which spontaneous recovery does not occur. Metz and Whishaw have demonstrated that it is relatively easy to train rats to cross a horizontal ladder, even following lesions to the sensorimotor cortex, pyramidal tracts, or spinal cord (Metz and Whishaw, 2002). Our own preliminary work training rats on a ladder showed that little recovery occurred during a 3-week delay period following spinal cord injury (indicating the feasibility of training rats after a delay period following spinal cord injury), but that animals do improve significantly over a 3-week training period, in which they traverse the ladder many times daily.

A number of aspects of the training process can be studied in this fashion, with potentially important implications for human training paradigms. In addition to studying the actual effects that a delay before training has on the benefits of training, one can

explore the underlying mechanisms of both the training process, and any change that may take place due to the delay period. This allows for detailed study of reflexes and muscle properties as they may relate to improvements in accuracy of stepping. Anatomical studies can be performed to identify which descending tracts are implicated in recovery, whether by 'taking over' functions of lost fibers, or by collateral sprouting. These studies can be done histologically, and by using electrophysiology (see Chapter 3), giving a combination of anatomical and physiological correlates of the behavioral changes produced by training. For instance, Weidner et al. (2001) identified sprouting from the ventral CST onto motoneurons pools in the spinal cord following lesions of the dorsolateral CST, and correlated this sprouting with the recovery of reaching and grasping abilities in the forelimb. Similarly, changes in behavior may be correlated to altered connections by monitoring the strength of evoked potentials when stimulating the motor cortex and recording EMG activity in the muscles of interest. One might also examine the expression of messenger ribonucleic acid (mRNA) and proteins (via in-situ hybridization and immuno-staining, respectively) known to contribute to neural plasticity in the spinal cord and brain. And finally, training could be combined with other interventions that increase plasticity and permissiveness to neurite growth, both to improve overall recovery, and to test whether any changes that take place due to the delay period are reversible.

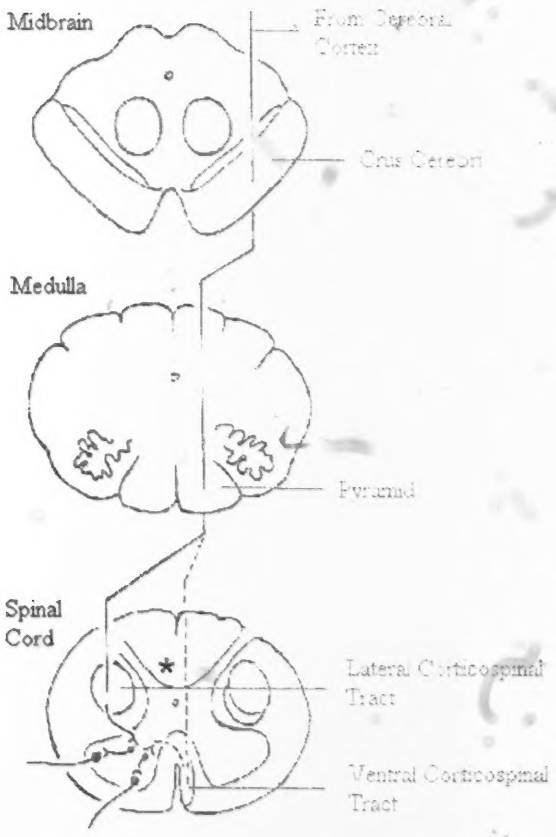

Figure 1 – The Human Corticospinal Pathways

The figure displays the course of the human corticospinal tracts. A single tract descends to the medullary pyramids, below which it splits into two distinct pathways. One decussates and descends in the lateral funiculus, while the other remains on the ipsilateral side and descends via the ventral funiculus. In the rat, the contralateral pathway descends along the dorsal column, pictured by * above. In the human, CST fibers terminate directly onto motoneurons and interneurons in the ventral grey matter of the spinal cord.

Picture taken from public website at: http://137.222.110.150/calnet/UMN/page2.htm

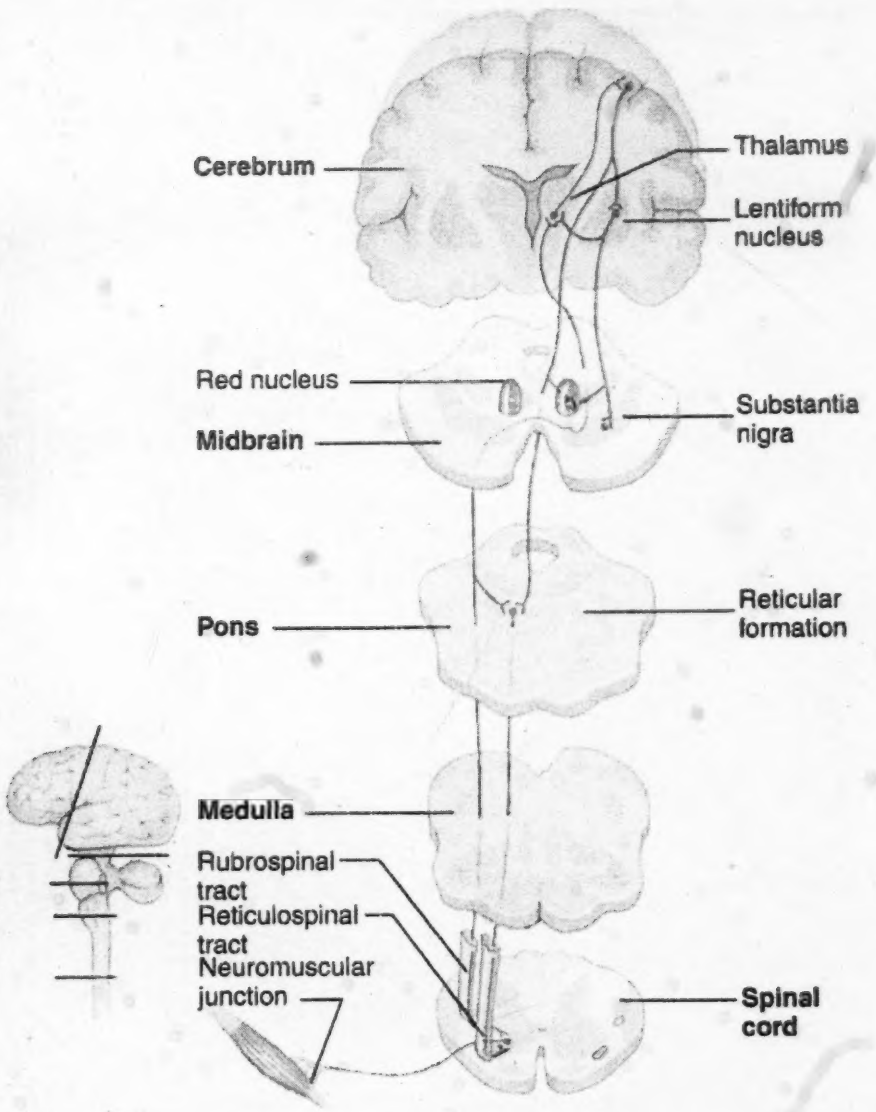

Figure 2 – The Red Nucleus and Rubrospinal Tract (RST)

The red nucleus receives inputs from the motor cortex. The fibers of the RST decussate immediately and descend contralaterally in the lateral funiculus. The RST terminates on interneurons in the ventral grey matter of the spinal cord, with no direct connections to motor neurons.

Picture taken from public website at: www.science.uwc.ac.za/physiology/neurology/neuro.cont.htm

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<Chapter 2>

Impairment of Functional Recovery Due to a Time Delay between Spinal Cord Injury and Motor Training

2.1 Introduction

Intensive daily training improves functional locomotor recovery following spinal cord injury (Barbeau and Rossignol 1987; de Leon et al. 1998; Muir and Steeves 1997; Wernig et al. 1995) and the benefits are retained for some time after training concludes (De Leon et al. 1999; Wernig et al. 1998). Clinical studies suggest that the timing of training onset may be important as a long delay period between injury and commencement of training seems to reduce the beneficial effects of treadmill training regimens (Wernig et al. 1995). This could have important implications for rehabilitation. While the effects of training appear to be more beneficial when training is implemented early after the injury, elucidation of the mechanisms underlying this phenomenon may help in the identification of therapies that make the spinal cord more responsive to training after a long delay period. However, due to the inability to accurately measure the lesion profile and control the training delay period in humans, clinical results are very difficult to interpret. Despite this, no animal model has previously been developed to assess the impact of a delay in locomotor training onset following incomplete spinal cord injury.

The unique challenges of human bipedal locomotion are difficult to emulate in a quadrupedal animal model, making direct comparison between the two problematic.

Spinal cord injuries that are anatomically analogous, such as destruction of the corticospinal tract alone, produce far less locomotor deficits in rats than in humans (Little et al. 1988). For instance, in humans, transection of the corticospinal tract alone is enough to produce a loss of all ability to walk (Nathan 1994). Although there is strong evidence to suggest that the human spinal cord contains central pattern generators for locomotion (Calancie et al. 1994; Dimitrijevic et al. 1998), studies using transcranial magnetic stimulation (TMS) have shown that corticospinal inputs provide part of the drive to ankle dorsiflexors during the swing phase of walking (Capaday et al. 1999; Petersen et al. 2001; Schubert et al. 1999). In contrast, during locomotion the corticospinal tract in quadrupedal animals is more involved in skilled movements and gait modifications (Beloozerova and Sirota 1993; Drew et al. 1996; Metz et al. 2000). After incomplete spinal cord injury involving damage to the corticospinal tract, rats display little to no deficit in simple overground walking, but have difficulty traversing a horizontal ladder (grid test) that requires accurate stepping (Little et al. 1988). Thus, in this study, we chose to look at accurate stepping in rats, a task that requires control from descending pathways that are similar to those used for walking in humans.

Unilateral transection of the corticospinal tract alone at the level of the pyramids produces an error when crossing the horizontal ladder of approximately 20% in the affected leg (Metz and Whishaw 2002; Z'Graggen et al. 1998). Because we desired a more robust deficit that could show a greater range of recovery, a more extensive lesion was required. The main descending tracts that mediate obstacle avoidance and accurate placement of the limbs in cats and rats are the corticospinal and rubrospinal tracts (McFadyen et al. 1999; Rho et al. 1999; Whishaw et al. 1998; Widajewicz et al. 1994).

Thus, partial or complete disruption of these two tracts results in varying degrees of functional impairment. To obtain a unilateral deficit we performed a dorsal quadrant lesion, aiming to unilaterally sever the entire corticospinal tract and the majority of the rubrospinal tract.

In summary, this study outlines the use of a rat model of locomotor training on a horizontal ladder to test how delaying the onset of training after a spinal cord injury by three months effects the recovery of accurate stepping in comparison to animals trained immediately after an injury. The advantage of examining the recovery of accurate stepping compared to overground locomotion is that the amount of training that the animal receives can be directly controlled as accurate stepping cannot take place in the rat's regular cage. A lesion of moderate severity was performed such that significant training induced recovery of accurate stepping could take place while allowing the animal to retain a large amount of mobility on a flat surface. The recovery pattern of descending (e.g. tactile and proprioceptive) and segmental (ground support response) reflexes were also examined to determine if they correlated with functional recovery in accurate stepping. The results obtained support the idea that functional motor recovery achieved through training is less effective when a significant delay period occurs between injury and training.

2.2 Methods

2.2.1 Training

All procedures were approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee. Thirty-seven Sprague Dawley female rats were studied. Animals were trained and tested on a raised horizontal ladder, 1 m in total length (testing field 0.8 m, allowing the animal space to maneuver on each end), with rungs approximately 1cm in diameter (**Figure 1A**). Spacing between rungs was between 3-8 cm, randomly allocated and rearranged regularly so animals could not memorize a pattern. Animals crossed freely, or were gently coaxed by the trainer, and a food reward was placed at the end of the ladder. Error rate was recorded as the number of misplaced steps over the total number of steps taken. Misplaced steps were counted as any steps in which the foot or toes descended between the rungs past the rung midpoint causing the animal to fail to achieve weight-support because the toes and ventral metatarsal pad did not land on the center of the rung, resulting in subsequent slippage (**Figure 1B**). These foot slips correspond approximately to values of 0-3 in the foot fault score outlined by Metz and Whishaw (2002). A training session consisted of 10 consecutive ladder crossings; animals were trained 3 times daily, 5 days/week for 3 weeks, which was found in preliminary studies in immediately trained animals to be sufficient for notable motor improvement. In a subset of animals (n = 8), training was extended to 6 weeks, but little improvement was seen in any animal past the first 3 weeks of training.

Prior to surgery, animals were pre-trained for one week on the ladder to become acquainted with the ladder and handler. Intact rats had little trouble navigating the ladder, and at the end of the first week the error rate was approximately 2% on average (**Figure**

4, day 0). Following surgery, rats were trained and videotaped daily. Error measurements in accurate stepping were calculated from video recordings every second weekday by a person not responsible for training and data collection, but not blinded to the training group. The error for each training day was calculated from the second training session that day, with the average obtained from the 10 runs across the ladder that session.

2.2.2 Surgery

A T8 partial laminectomy was performed under subcutaneous hypnorm/versed (0.635mL/kg and 6mg/kg) or sodium pentobarbital anesthesia (35-40mg/kg). A small incision was made in the dura mater and lidocaine was applied to the spinal cord. A fine razor, broken and filed down to 1.5mm width, was then inserted 1mm deep at the midline, and pulled laterally to unilaterally sever the dorsal and dorsolateral funiculi. Animals were allowed to recover from anesthesia on a water-circulating heat blanket, and 0.05mg/kg buprenorphine was given for analgesia.

2.2.3 Delay Protocol

Following surgery, animals began training immediately (3-4 days post-operative) or after a 3-month delay period. Age matched controls were also studied (n = 6), in which animals were kept in their cages for 3 months before the surgery, then trained immediately afterward to account for the possibility of an aging effect on training. The average weights and ages of the animals at commencement of training were as follows: Immediate Group - 220g; 10 weeks old. 3-Month Delay Group – 330g, 22 weeks old.

Age-Matched Immediate Group – 310g, 22 weeks old. All animals were considered to be mature adult rats.

2.2.4 Histology

After the conclusion of training, animals were transcardially perfused with saline, followed by 4% paraformaldehyde fixative solution. Spinal cords were dissected out, and placed in paraformaldehyde solution overnight, then transferred to a 30% sucrose solution for dehydration. Fixed, dehydrated tissue was frozen in isopentane at -40°C, and stored in a -80°C freezer. Sections of spinal cord from approximately T6-T12, which surround the T8 lesion site, were sliced into 25μm sagittal sections on a cryostat, mounted in serial sections on slides, cresyl violet or silver-stained, and cover slipped. Every second section was taken.

2.2.5 Reconstruction of Lesion Site

To estimate the extent of the lesion, serial parasaggital sections of spinal cord, including and surrounding the lesion site, were prepared and stained as above (**Figure 2A-D**, dorsal surface of the cord is at the top) in a similar manner to that done by Fouad et al. (2001). Under light microscopy, landmarks such as the central canal and the gray-white matter profile were used to identify the approximate medio-lateral location of a particular slide on a representative horizontal section (**Figure 2E**). For example, the parasaggital section in **Figure 2A** is composed entirely of white matter and thus, was taken from a very lateral section of the spinal cord, marked as **A** in **Figure 2E**. In **Figure 2B**, a thin band of gray matter appears on the dorsal aspect of the cord whose dorso-

ventral extent is indicated by the white band for clarity. Section **B** is matched to the appropriate medio-lateral location on the horizontal schematic where a relatively small proportion of gray matter appears near the dorsal surface to the left of section **A**. In contrast, section **C** displays a thicker band of gray matter toward the center of the cord and its proportion of gray to white matter matches that on the location marked **C** in **Figure 2E**. Finally the central canal is clearly visible in section **D** (asterisk) which is located in the midline of the cord as shown in **Figure 2E**. Typically, the midline and most lateral parasaggital sections on each side of the spinal cord were identified first and remaining sections were then spaced according to their grey-white matter profile.

The size and location of the lesion was mapped by measuring the maximal extent of dorso-ventral damage, observed as disruption of white matter tracts and scar formation, in each parasaggital section and transferring this information to the appropriate medio-lateral location on the 2-D horizontal representation (**Figure 2F**). For example, in **Figure 2A** and **B**, no white matter damage is apparent, while the arrows in **Figure 2C and D** indicate the extent of dorso-ventral damage which extends to the base of the gray matter in **C** and mid-way through the dorsal funiculi in **D**. The corresponding extent of damage is represented by the arrows on the horizontal representation in **Figure 2F**. The entire extent of dorso-ventral damage to the spinal cord was then determined by joining together all points of maximal ventral damage for each parasaggital section on the 2-D horizontal schematic.

In a final step, the location of the corticospinal and rubrospinal tracts, as obtained from retrograde and anterograde labeling studies (Brosamle and Schwab 1997; Midha et al. 1987; Tator et al. 1984), were drawn on the horizontal schematic (**Figure 2F**). The

area of each tract that was compromised by the lesion was measured by placing a grid over the schematic and counting the number of squares that were within the lesioned area. The amount of damage to each of these tracts was then expressed as a percentage of the total area of the tract, i.e., a score of 100% indicated that the entire tract was damaged. The combined area of damage to the ipsilateral rubrospinal and corticospinal, and contralateral corticospinal tracts was used as an indication of 'total tract damage'. The amount of tract damage for each individual tract, all three tracts combined or a combination of two tracts were then compared to the initial behavioral deficit and performance during training. Formation of fluid-filled cysts sometimes complicated the estimation of tract damage as such cavitation can cause compression of white matter without dramatically compromising tract function (Olby and Blakemore 1996). In such cases, we tried to determine the extent of integrity of the remaining fibers, and reduced the damage estimate if it appeared that the fibers were displaced but not damaged. For all histology done in this study, the person performing lesion reconstruction was blinded regarding which group each animal was in.

2.2.6 Reflexes and Muscle Stiffness

2.2.6.1 Tactile Placing Response

The recovery pattern of descending (e.g. tactile and proprioceptive) and segmental (ground support response) reflexes were examined to determine if they may have contributed to the recovery of accurate stepping. To examine the tactile placing response, the animal was held upright, with the hindlimb pendant. The dorsum of the paw was lightly brushed against a force transducer plate *without* producing movement of the ankle

joint. A positive tactile placing response was considered to have occurred when the leg made a stepping motion over the plate. The force required to elicit the reflex was recorded in each leg, and maximum threshold of 10g was set. Above this threshold, responses were taken to be proprioceptive (below).

2.2.6.2 Proprioceptive Placing Response

Similar to the tactile placing response the dorsum of the paw was brushed against a force transducer, except that a visible movement of the ankle joint (approximately 10°) was produced. This reflex, therefore, had a higher force threshold than the tactile placing response, and was easy to record post-operatively, as the tactile placing response was abolished in most animals. Minimum threshold for this response was taken as 10g (or approximately 100mN). Below this threshold, responses were taken to be tactile responses.

2.2.6.3 Ground Support Response

The animal was held upright, with the hindlimb pendant over a scale. The plantar surface of the paw was slowly placed on the scale, and the animal was lowered. The maximum force applied on the surface of the scale was recorded for each leg.

2.2.6.4 Muscle Stiffness

Passive muscle properties (stiffness) of the leg were also examined to test if changes in muscle stiffness contributed to the differences in motor recovery observed between the immediate and 3-month delay animals. Animals were anaesthetized with

isoflurane, placed in a supine position, and the foot was affixed to a motorized arm such that movement of the arm displaced the ankle joint by 18°. The arm was set to oscillate at different frequencies, ranging from 0.5 to 20Hz, and passive stiffness of the muscles around the ankle and knee as a whole was calculated as the peak resisting force multiplied by the peak displacement, then divided by the angle of the moment arm (in radians), yielding a value for stiffness in units of Nm/rad.

2.2.7 Statistics

Group averages were compared using a Student's t-test. Linear regression was used to calculate goodness of fit between initial error or % improvement in accurate stepping and the anatomical extent of the lesion. In addition, the slopes of these regression lines were compared for statistical difference between the immediate and 3-month delay animals. All statistics were calculated using Sigmaplot software. Comparison of regression line slopes was performed using a β-test, by using standard error and the appropriate t-distribution to determine the confidence interval for the slope of the regression line with the confidence interval = $(\beta_1 - \beta_0)/t_{(n-2)}(SE)$.

2.3 Results

2.3.1 Lack of spontaneous recovery in accurate stepping

Following unilateral lesions of the dorsal quadrant, rats quickly recovered the ability to walk on a flat surface (Kaegi et al. 2002; Little et al. 1988) even though accurate stepping on the horizontal ladder was impaired. On the ladder, a large range of error values were apparent prior to training (initial error) in both the immediately trained

animals tested at 3 days post-surgery and the 3-month delay animals tested at 90 days post-lesion (**Figure 3**). The average initial error for the immediate group was 61.8 +/- 23.3% (n = 21), and similar to that of the 3-month delay group, which was 64.3+/- 17.2% (n = 16), not significantly different (p = 0.72). This suggests that impairments in accurate stepping did not improve spontaneously over time as initial performance error in the delay group, which were allowed to move freely within their cages for 90 days but not trained, were similar to the immediately trained animals tested 3 days post-injury. The variability of the initial error within the groups reflects variability in lesion size and profile, discussed below in Section 2.3.3.

2.3.2 Effect of delay on training response

The 3-month delay period decreased the improvement on the ladder produced by training the animals 3 times/day, 5 days/week for 3 weeks. The initial error, after injury but before training (day 1, **Figure 4**) was identical for both groups (61.8 ± 23.3% for immediate, 64.3 ± 17.2% for delay, p = 0.72). However, by day 3, a trend developed in which the immediate group displayed a decrease in error rate as training went on. The 3-month delay group also improved over time, but to a lesser extent. The spread between the groups appeared to increase over the course of training, and by the end of the 3-week training period (day 19, **Figure 4**) was statistically significant (23.8 ± 24.9% for immediate vs. 51.8 ± 30.5% for delayed, p = 0.003). Animals trained for 6 weeks with no delay (immediately trained group, n = 8 animals) showed no substantial improvement in error past the third week of training (n = 8, 0.2% difference in average error between week 3 and 6, p = 0.47). In addition, the reduction in error in the 3 month group reached a

plateau after the second week of training. The age-matched control group showed an improvement not significantly different from the non-age-matched animals trained immediately after injury (age-matched = 67.7% improvement, non-age-matched immediate = 59.0%, p = 0.89), and was therefore included in the overall immediate trained group.

2.3.3 Comparison of accurate stepping and lesion size

To effectively compare the two groups, we needed to ascertain that the histological profiles of the lesion were comparable. The histological profile of the spinal cord lesion for each animal is displayed in **Table 1** and indicates the percentage of damage in each of the ipsilateral corticospinal and rubrospinal tracts (iCST and iRST) and the contralateral corticospinal tract (cCST), in addition to the corresponding initial error and % improvement in accurate stepping (see Methods for details on lesion reconstruction and calculation of stepping error). The total damage to the spinal cord (i.e., gray matter plus white matter) was found to be a less reliable predictor of performance than the destruction of the three descending white matter tracts and thus was not used. Comparison to all three tracts combined (All 3) was also much better than any tract individually or combination of any two tracts. The average damage sustained by the 3-month delay group was somewhat greater than that of the immediate group, although the differences were not significant on a Student's t-test (p-values: iCST = 0.35, cCST = .83, iRST = 0.28). In general, animals with more than 80% combined damage in the three descending tracts achieved very little (< 20%) improvement in response to training.

The estimated extent of the lesion (% of total damage in the 3 tracts) was plotted against initial error (**Figure 5**) and % improvement (**Figure 6**). The lesion profile correlated well with the initial error rate for both the immediate and delayed group with the coefficient of determination (r^2) of the linear regression being 0.72 and 0.73, respectively. The slope of the regression line for the immediate group was significantly higher than that of the 3-month delay group (**Figure 5**, 0.97 vs. 0.54, $p = 0.02$) because immediately trained animals displayed greater initial error compared to their 3-month delay counterparts for lesions that were ≥ 65% (see Discussion). Despite this, after 3 weeks of training, the immediate trained group improved their ability to accurately step across the horizontal ladder after three weeks of training to a greater extent than the delay group. As shown in **Figure 6** the regression line for the %-improvement of the immediate trained group is well above the regression line for the 3-month delay group for all values of total tract damage (x-axis). This indicates that for a similar lesion profile, the immediate trained group achieved much greater improvement (≈ 50%) than the 3-month delay group after three weeks of training (average improvement: immediate group = 61.2%, delay group = 30.1%). The larger initial error in immediately trained animals with > 65 % tract damage may have provided more room for improvement, and thus partly explained the greater improvement with training. However, in animals with <65% tract damage that had identical initial errors (49.9% immediate, 51.1% delay, $p = 0.85$), the immediately trained group still showed a significantly greater % improvement in stepping error, compared to delayed trained animals (immediate group: 78.3%, delay group: 47.2% improvement, $p = 0.001$). Thus, the delay itself is the primary reason for the poorer performance.

2.3.4 Animals further matched by lesion profiles

Although there were no statistically significant differences in the histology profiles of the two groups, we analyzed the performance data versus the lesion profile for a subset of animals that had less than 80% total destruction of the 3 tracts in order to match the two groups better histologically (n = 18 immediate; n = 10 delay). This resulted in improved similarity in the average lesion profile for the two groups **(Table 2)**. While the values for damage to each descending tract were impressively similar (iCST: immediate = 74.4%, delay = 75.8%, p = 0.90; cCST: immediate = 14.7%, delay = 14.6%, p = 0.984, iRST: immediate = 53.8%, delay = 57.8%, p = 0.789), a large significant difference *still* remained in the % improvement after 3 weeks of training **(Figure 7B**; 72.5% for the immediate matched group vs. 42.3% for the delayed matched group). Likewise, the matched immediate group achieved a significantly lower % error after three weeks of training (from 59.0% to 19.5%) compared to the delay group (from 54.3% to 34.6% error). The plots for matched lesion vs. initial error **(Figure 7A)** and matched lesion vs. % improvement **(Figure 7B)** resemble those of the entire group of animals. Interestingly, the discrepancy in regression slope remains significant for the lesion vs. initial error for the matched groups (immediate = 1.07, delayed = 0.46, p = 0.001, see Discussion).

2.3.5 Comparison of stepping strategy

We observed that there were two strategies that could be used for stepping on the ladder; the lesioned limb could be placed ahead of the control limb on each step, termed leading step, or it could be placed on the same rung as the control limb, termed trailing

step. To ensure that differences in stepping strategy did not account for the differences in accurate stepping between the groups, we observed the stepping strategy in a subset of training sessions (n = 10) for 6 animals, one on the first day of training (no difference in accurate stepping between groups) and one after 3 weeks of training (significant difference in accurate stepping). At the start of training, the immediate group took leading steps 75.7% of the time vs. 70.7% for the delay group (t-test, $p = 0.21$), while at the end of 3 weeks of training the values were 82.6% leading for the immediate group, and 79.9% for the delay group (t-test, $p = 0.58$). Thus, the stepping strategy was very similar between groups, and does not correspond to changes in accurate stepping or the discrepancy in accuracy between groups.

2.3.6 Time course of recovery of descending and segmental reflexes

Although our data described above suggest that functional recovery is mediated by alterations in the activation of spared descending systems controlling accurate stepping, we analyzed some basic descending and segmental stepping-like reflexes which may also have contributed to the improved performance (Thompson et al. 1998). In addition, we wanted to test if the effects of ladder training had any carry over to other motor systems.

2.3.6.1 Ground support response

Immediately after the spinal cord lesion, the ground support response decreased in the leg ipsilateral to the spinal lesion in the immediate group by 14% three days after injury and by 7% seven days after injury (Fig. 8A, open symbols, n = 9) compared to pre-

lesion values (day 0). Likewise, a similar decrease of 11% was seen in the delay group three days after injury (Figure 8B, open symbols, n = 5), but seven days after injury the ground support response was restored to 7% higher than the day 0 value. However, the increase was less than the increase observed in the control leg (17%), which was partly attributed to weight gain. Interestingly, in both the delayed and immediate groups, the ground support reflex in the affected leg recovered to values above that of the control leg by day 28 after injury, even though the delay group was not being trained during this period. This indicated that the training had little effect on the ground support response because even without training, a similar change occurred in the 3-month delay group as was observed in the immediate group during training. Moreover, this suggests that the improvement observed in accurate stepping in the delay group was not mediated by improvements in reflexes as the ground support response did not undergo any further changes during the delayed group training period, i.e., an 8% bilateral increase occurred during the 3 week training period that was attributed to weight gain. The absence of a reduction in support response observed in the control leg at day 3 (contralateral to lesion site, **Figure 8**, closed symbols) indicates that the lesion was generally restricted to one side of the spinal cord.

2.3.6.2 Tactile and proprioceptive placing response

The tactile and proprioceptive placing responses, which involve a compensatory dorsiflexion following contact upon the dorsum of the paw, seemed the most likely reflexes to be involved in the improvement on the ladder, a stepping task. The cortically-mediated tactile placing response (Amassian et al. 1972) was abolished in virtually all

animals after injury, in that a contact force of 10g or less to the dorsum of the paw produced a stepping response before the lesion, but was no longer able to do so after the lesion (n = 24/27 animals). In these animals the tactile placing response never returned, despite the recovery on the ladder walking task. The only animals that had a preserved contact placing response were animals with 35% damage or less to the ipsilateral CST (n = 3/27), and this response did not undergo any marked changes in these animals with training, as in Basso et al. 2002.

The proprioceptive response, which is believed to have descending and segmental components, (De Ryck et al. 1992) remained intact after the injury. However, the threshold for this response in the leg ipsilateral to the lesion increased soon after injury in both the immediate group (45% at day 3 and 38% at day 7, **Figure 9A,** open symbols, n = 9) and the delay group (39% at day 3 and 65% at day 7, **Figure 9B,** open symbols, n = 5) compared to pre-lesion control values (day 0). The threshold decreased and returned to approximately pre-lesion values over 3 weeks for both groups, irrespective of whether the animals were being trained (immediate group) or not (delay group). Thus, the recovery of the proprioceptive reflex does not correlate to performance, as the immediately trained animals were improving rapidly on the ladder before the reflex recovery occurred, while the delayed group made no such improvements until long after the reflexes were restored to pre-lesion levels. When the delayed group was trained, the improvement in accurate stepping, while not as impressive as that of the immediate group, occurred without any corresponding changes in the proprioceptive threshold. Upon completion of training at 104 days post-injury, the delay group values were 109% (control leg) and 104% (lesioned leg) of values recorded 28 days after the injury.

Reflexes were also analyzed for another subset of delay animals (n = 11). Although reflexes were not recorded at all time-points as the other groups, the overall trend was similar, with lesioned legs displaying a transient increase and decrease in proprioceptive thresholds and ground support responses, respectively, 7 days after injury with both values returning to near pre-lesion levels at 28 days post-injury.

2.3.6.3 Passive muscle stiffness

Passive muscle stiffness was tested at the end of the training regime in 4 animals to rule out changes in static muscle properties as a major factor contributing to the difference in functional recovery achieved in the two groups of animals. No significant differences were seen between the lesioned and unlesioned legs in any of the animals, either delayed-group or age-matched immediate (average stiffness = 23.3 Nm/rad for lesioned leg vs. 23.1 Nm/rad for intact leg). This indicates that no dramatic changes in passive muscle properties occurred that were specific to the affected leg and that differences in muscle stiffness between the immediate and delay group were not responsible for the differences in eventual motor recovery achieved.

2.4 Discussion

The results demonstrate that recovery of the ability to step accurately on a horizontal ladder following injury to the dorsal quadrant of the spinal cord does not occur spontaneously. Rats that experienced a 3-month delay exhibited the same initial error, on average, as rats that began training 3 days after injury indicating that overall no substantial improvement occurred during the delay period. The delay group exhibited a

marked decrease (50%) in improvement with training compared to the rats trained immediately after injury. Although some variability in improvement was apparent within groups, the broad trend indicates that the delay period makes animals less responsive to rehabilitative training. A large part of this variability was due to the lesion size, and when this was compensated for by matching animals according to lesion profile, the difference in improvement between groups remained. The recovery of accurate stepping on a horizontal ladder was not associated with changes in stepping-like reflexes. Although some reflex changes occurred during the period of improvement in rats trained immediately after injury, similar reflex changes were apparent in rats that were not undergoing training at that time (i.e., the 3-month delay group), indicating that the reflex changes did not relate to training, or to training-induced recovery.

2.4.1 Comparison between Groups: Initial Error

The slope of the regression line for total tract damage vs. initial error is steeper for the immediate group than the delay group (**Figure 5**). This difference is significant even when animals were further matched for histology. Part of the reason for this discrepancy between immediate and delay group animals is that the animals with the largest lesions (i.e., > 70% damage) in the immediate group displayed significantly higher initial error than their 3-month delayed counterparts. A few factors may have contributed to the difference between the groups. It is possible that the animals with severe injuries that were trained and tested immediately (3 days) were not yet fully recovered from the adverse side effects of the injury and surgery, contributing to a worse performance than would be expected from a lesion of that size. Also, if any swelling remained in the spinal

cord, it could have contributed to a lack of appropriate activity or aberrant activity, hampering the animal's ability to step accurately. Finally, excessive extensor activity, which has been reported during the first two weeks following dorsal spinal cord injury (Kaegi et al. 2002), may have contributed to problems accurately placing the hindlimbs. Because immediately trained animals with moderate and mild lesions (i.e, < 70% tract damage) did not display initial error values higher than 3-month delay rats with similar lesions, the most likely explanation is that transient effects from a large lesion in the immediate group hampered the ability of these rats to accurately step on the ladder.

Admittedly, it is impossible to entirely discount spontaneous recovery. Due to the nature of our training/testing paradigm, it is impossible to ascertain what the initial error of the delay group *would have been* if the animals had been tested sooner after injury, as any actual testing constitutes a training session which could consequently alter the results of the group supposedly not trained for 3 months. Despite this, we are confident that the very similar initial error on the first day of training (**Figure 3**) in the large group of animals studied demonstrates that no significant spontaneous recovery occurred, especially for rats with < 70% total tract damage. Thus, the improvements in stepping performance can be, for the most part, attributed to daily motor training.

2.4.2. Comparison between groups: % improvement

The animals trained immediately displayed a greater improvement (%-improvement as decrease in error divided by initial error) than the delay group. This is apparent both in the %-improvement (61.2% for immediate vs. 30.1% for delay) and the final error level reached (23.9% for immediate vs. 50.6% for delay). Because the initial

error was very similar for the entire group of animals (61.8% for immediate vs. 64.3% for delay), comparisons of the %-improvement correlated directly to comparisons of the final error. The animals selected for the matched histology groups displayed a similar trend. The overall initial error was slightly higher in the immediate group compared to the delay group for the histology-matched animals (58.5% for immediate; 55.9% for delay). Correspondingly, the immediate group showed a greater %-improvement (72.5% for immediate vs. 42.3% for delay) and also reached a lower level of final error (16.8% for immediate vs. 32.2% for delay).

In the matched group, the greater improvement achieved by the immediate trained animals could have been due to the higher initial error providing more room for the animals to improve upon. However, in the matched group, when only those animals with very similar initial errors were compared, i.e., animals with < 65 % total lesion (immediate average = 49.8% initial error, n = 16, delay average = 51.1% initial error, n = 7, $p = 0.85$), then there was still a substantially greater improvement with training in immediate, compared to delayed trained, animals (immediate average = 78.3% improvement, delay average = 47.2%, $p = 0.001$). Thus, differences in initial error do not account for the improvement.

2.4.3 Variability in lesion reconstruction

Some variability exists in our lesion reconstruction methods. Although we identified the amount of damage done to 2-dimensional spinal cord reconstructions, and located the tracts of interest based upon numerous labeling studies in the literature, our representation may not be entirely accurate. Also, compression of cord tissue due to fluid

accumulation or other factors may result in misrepresentation of the extent of the damage. The immediate animals were kept for a few months before they were sacrificed so that all histology in the two groups was done at approximately the same time after the lesion, avoiding time-related changes in the state of the injured spinal cord. A more accurate method for the quantification of damaged and spared fibers would be retrograde labeling of axons below the lesion site and subsequent counting of cell bodies in the cortex and red nucleus. However, despite the inherent variability, we were able to achieve a reasonable degree of correlation between our estimates of the extents of the lesions and the behavioral effects incurred by them. Moreover, the large size of the sample populations helps to compensate for the variability inherent in the procedure, as it is similar in both groups.

It is interesting to note that the y-intercepts for the plotted regression lines for the initial error vs. lesion extent for the delay group in **Figure 5** and **Figure 7A** are approximately 30%, not zero as one would expect based on a linear decrease in error with lesion size. However, damage to dorsal ascending columns, not accounted for in our correlations, can contribute to errors in accurate stepping (Goldberger 1988). Also, the relationship may be non-linear for very small and very large lesions, as small amounts of damage to relevant areas may have a large impact. Regardless of differences in lesion vs. initial error, it is clear that the improvement is greater in the immediate group for all comparable lesion sizes.

2.4.4 Reflex Results

Alterations in descending and segmental reflexes and muscle stiffness did not correlate with functional recovery. Because accurate stepping requires control from descending systems, descending and segmental reflexes are unlikely to be heavily involved in recovery of accurate stepping after spinal injury. While the tactile and proprioceptive placing responses bear some resemblance to the stepping motion on the horizontal ladder, there is no relation of improvement in accurate stepping to the reflexes studied. This is clear because the timeline of modulation of reflex activity following spinal cord injury is similar for the immediate and delay groups despite the fact that one group is being trained while the other is not. This indicates that training has little impact on these reflexes, and that the reflexes have little impact on improvement in accurate stepping. However, it should be noted that for simplicity the reflexes were studied in a stationary position rather than during walking, and that context-dependent modulation of reflexes may still have occurred (Stein 1995).

2.4.5 Mechanisms of training-induced recovery

Accurate placement of the hindlimb is not visually mediated. On the ladder, the rat is unable to see its hind feet, and thus must predict where to place them based on proprioceptive information from the forelimbs, the intact hindlimb or based upon remembered visual information. This information is then used to make accurate movements that correspond to this information. The lesion seems to interrupt this information relay, preventing accurate placement due to the inability to accurately act upon that information. While damage to ascending pathways probably has a negative

impact on accurate stepping, damage of the dorsal ascending columns alone is probably insufficient to produce significant deficits in rats walking on the ladder (Goldberger 1988). Because descending control is so important in this paradigm, a useful way to think of the cause of error and subsequent improvement is in terms of loss of descending projections from the planning and integrating areas of the motor cortex to the motor output cells (interneurons and motoneurons) in the spinal cord. Functional recovery requires improved connectivity between these centers.

The unmasking and synaptic strengthening of existing connections (Bach-y-Rita 1990), rather than sprouting, is probably the main mechanism of the observed functional recovery. It is unlikely that functional sprouting can occur quickly enough to mediate the rapid improvement seen in the first week of training in the immediate group. Previous work has shown that while retraction bulbs form on severed axons within a day after injury, sprouting is not detectable until 3 weeks after injury (Hill et al. 2001). Thus, it is most likely that reorganization of existing descending pathways is responsible for improvement. Indeed, if all or the majority of the 3 tracts (iCST, cCST and fRST) were destroyed, little to no improvement was observed in those animals. Conversely, if damage to these tracts was very limited (< 20%) there was little or no initial error on the ladder; even when substantial damage was sustained by the dorsal ascending columns (immediately dorsal to the dorsal CST, data not shown). Because we did not see a strong correlation between improvement and any individual descending tract, it seems likely that the compensatory reorganization involves all of them (if spared), although it is not necessarily limited to just these.

Improvement in accurate stepping correlated with sparing in the CSTs and iRST, indicating that sparing of these fibers is important, and may be the primary location of fast reorganization leading to functional recovery. In addition to spared CST and RST fibers, a number of prospective pathways exist which could contribute to training-related improvement. Extensive proprioceptive systems exist in the rat spinal cord (Miller et al. 1998), yielding indirect, multisynaptic connections between the motor cortex and spinal motor neurons. Crossed fibers from the spared CST, which exist as a relatively small portion of the descending CST < 5%, (Satomi et al. 1988), may also play a role in functional recovery. The reticulospinal and vestibulospinal systems are not normally involved in skilled movements like accurate stepping (Matsuyama and Drew 2000), but may undergo a partial shift of function following injury.

2.4.6 Physiological Effects of Delay

The reduced benefits of training after a time delay can be attributed to the changes that occur in the brain and spinal cord during the time delay. Specifically, we suggest that the immediate group is being trained for a specific behavioral function when the synapses are most plastic. The delay group gets no such timely behavior-specific training, and thus reorganization of synapses in the cortex, red nucleus and spinal cord that is not task-specific for ladder walking takes place, and thus, is unlikely to produce changes that lead to task-specific ladder walking improvements. By the end of the delay period, a large degree of reorganization has likely occurred, including the formation of synapses that may serve functions not related to accurate stepping, and is not easily reversible (Tillerson and Miller 2002). This can be tested by treating animals with therapies that

alter CNS plasticity such as use of IN antibodies, shown to increase reorganization of descending motor pathways (Raineteau et al. 2002). An increase in task-specific plasticity should correlate with improvements in functional recovery, especially for the delay-trained group.

2.4.7 Functional Implications

Our results indicate that it is best to begin training as early as possible following incomplete spinal cord injury. In some studies of rehabilitation following cerebral ischemia, researchers found that training too early actually increased motor deficits (Risedal et al. 1999). However, in stroke, the ischemia causes necrosis and apoptosis of cell bodies. Further metabolic strain, in an area of hypoperfusion, may incite more cells to undergo apoptosis (Stadler et al. 2001). In addition, reperfusion injury (Manabat et al. 2003) contributes to damage to cell bodies in the cerebral cortex, and perfusion may be further increased locally by the elevated activation of the cells (James et al. 1968). In spinal cord injury, damage to the axons is more important, and further insult does not induce apoptosis of neurons (Kwon et al. 2002). Moreover, increased activity has little effect on local metabolic activity at the lesion site, especially in glial cells that actually do undergo apoptosis. In our studies, the motoneurons and relevant interneurons involved in hindlimb stepping are not near the lesion site, so will not be directly affected by local factors such as inflammation. Thus, training immediately after spinal cord injury, when feasible, may be optimal to improve functional recovery via motor training.

3-month Delay Group (n = 16)						
Rat	***i*CST%**	***c*CST%**	**RST%**	**All 3 %**	**% Initial Error**	**% Improve**
C51	22.2	0.0	22.2	14.8	40.0	59.0
C50	100	83.3	77.8	87.0	72.3	57.1
C40	100	44.4	44.4	63.0	58.1	0.0
C52	61.1	10.0	83.3	51.5	38.6	68.9
C44	100	100	77.8	92.6	86.7	0.0
C46	100	33.3	100	77.8	76.5	6.8
C38	100	90.0	100	96.7	90.1	1.8
D57	100	20.0	100	73.3	66.1	29.2
D58	100	80.0	100	93.3	89.3	0.0
D59	100	45.0	100	81.7	75.8	0.0
D61	100	85.0	100	95.0	71.2	0.0
C48	55.0	0.00	0.00	18.3	45.0	33.7
C53	90.0	10.0	22.2	40.7	53.0	77.7
C54	35.0	0.00	77.8	37.6	62.3	61.2
C56	100	16.7	100	72.2	59.2	56.4
D62	94.4	11.1	27.8	44.4	44.2	29.8
Total	**84.9**	**39.3**	**70.8**	**65.0**	**64.3**	**30.1**
Immediate Group (n = 21)						
Rat	***i*CST%**	***c*CST%**	**RST%**	**All 3 %**	**% Initial Error**	**% Improve**
9	60.0	10.0	100	56.7	82.7	47.3
A12	100	5.0	77.8	60.9	71.7	84.9
A22	100	10.0	100	70.0	90.2	10.2
A13	100	10.0	100	70.0	92.9	36.4
A2	100	100	88.9	96.3	95.1	28.3
A18	50.0	22.2	0.00	24.1	51.0	85.9
10	40.0	0.00	0.00	13.3	39.3	75.8
A9	72.2	11.0	33.3	38.9	30.4	75.7
A23	100	0.00	0.00	33.3	48.0	89.6
13	65.0	11.1	72.2	49.4	61.5	85.2
11	100	33.3	27.8	53.7	65.5	80.2
A17	100	88.9	16.7	68.5	90.9	72.9
A5	91.7	11.1	27.8	43.5	29.7	76.1
12	80.0	5.6	45.0	43.5	49.8	91.0
J2	44.4	0.00	55.6	33.3	48.6	75.5
*E2	61.1	11.1	77.8	50.0	46.0	51.7
*F1	100	50.0	100	83.3	91.0	15.0
*F10	100	33.3	100	77.8	91.2	84.3
*E6	22.2	0.00	100	40.7	50.0	86.6
*F6	50.0	0.00	55.6	35.2	40.9	90.9
*F8	77.8	16.7	33.3	42.6	31.3	78.0
Total	**76.9**	**20.4**	**57.7**	**51.7**	**61.8**	**61.3**

Table 1 – Histological and Performance Profile of Lesioned Animals
The lesion profile of each animal included in the study. The numbers displayed are estimates of the proportion of damage (i.e., 100% = total tract damage) to the ipsilateral CST (iCST), contralateral CST (cCST) and the ipsilateral rubrospinal tract (RST) and all 3 tracts combined (All 3). Initial error represents the proportion of erroneous steps taken on the first day of training [(miss steps/total steps) x 100%. % Improvement indicates [(initial error-final error)/initial error x 100%]. The asterisk denotes animals in the immediate group that were aged for 3 months prior to injury, in order to match the age and condition of the 3-month delay group.

	*i*CST%	*c*CST%	RST%	All 3 %	% Initial Error	% Improvement
3-month (n = 10)	75.8	14.6	57.8	49.4	55.9	42.3%
Immediate (n = 19)	74.4	14.7	53.8	47.7	58.5	72.5%
p-values	0.900	0.984	0.789	0.818	0.739	0.002

Table 2 – Group Averages for Closely Matched Subset
A subset of animals was selected to maximize similarity in the histological profile between the immediate and 3-month delay group. 10 animals were selected from the 3-month delay group, and 19 animals from the immediate group, all with < 80% total damage. A Student's t-test was performed to test the difference of the means of the two groups. No differences were apparent in damage to any of the tracts studied, nor the initial error, however the improvement was significantly greater in the immediate group than the 3-month delay group ($p = 0.002$).

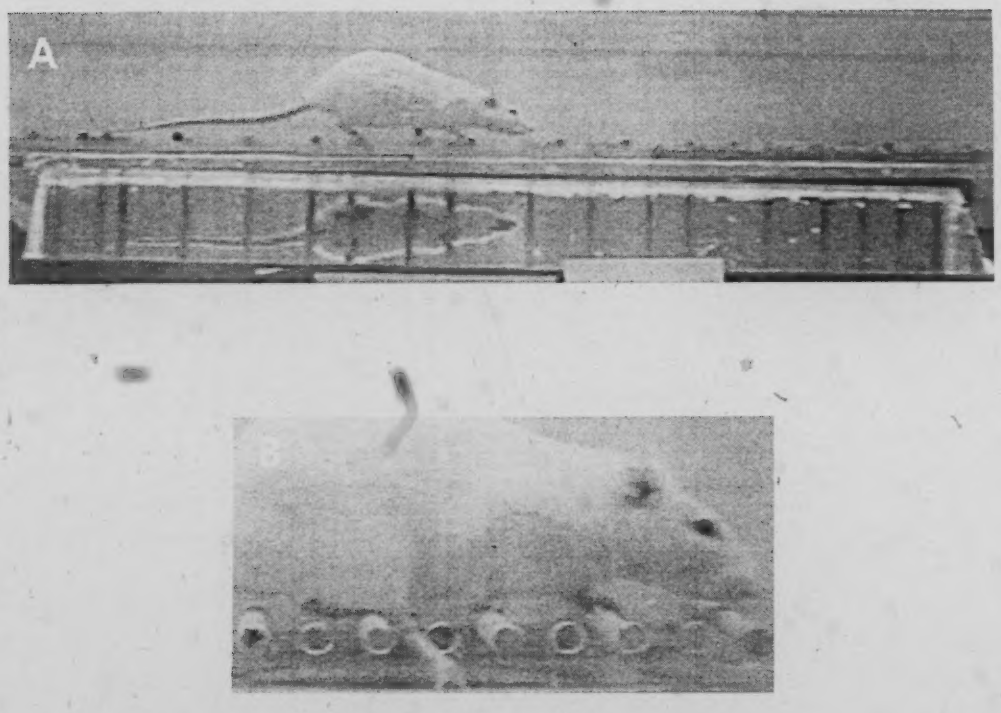

Figure 1. Demonstration of Horizontal Ladder and Stepping Error
(A) Side view of rat traversing the 80cm horizontal ladder. An angled mirror placed under the ladder allows viewing of the ventral aspect of the rat and ladder. Rats cross freely to reach a holding pen on one end of the ladder, but may also be encouraged with food rewards or gentle coaxing. The spacing between the rungs varies randomly from 3-8cm; rungs are 1cm in diameter. **(B)** Close-up of rat making an error with its right hind paw. An error in stepping is defined as any slippage of the paw between the rungs extending beyond the midpoint of the rung that results in the failure of a weight-bearing step.

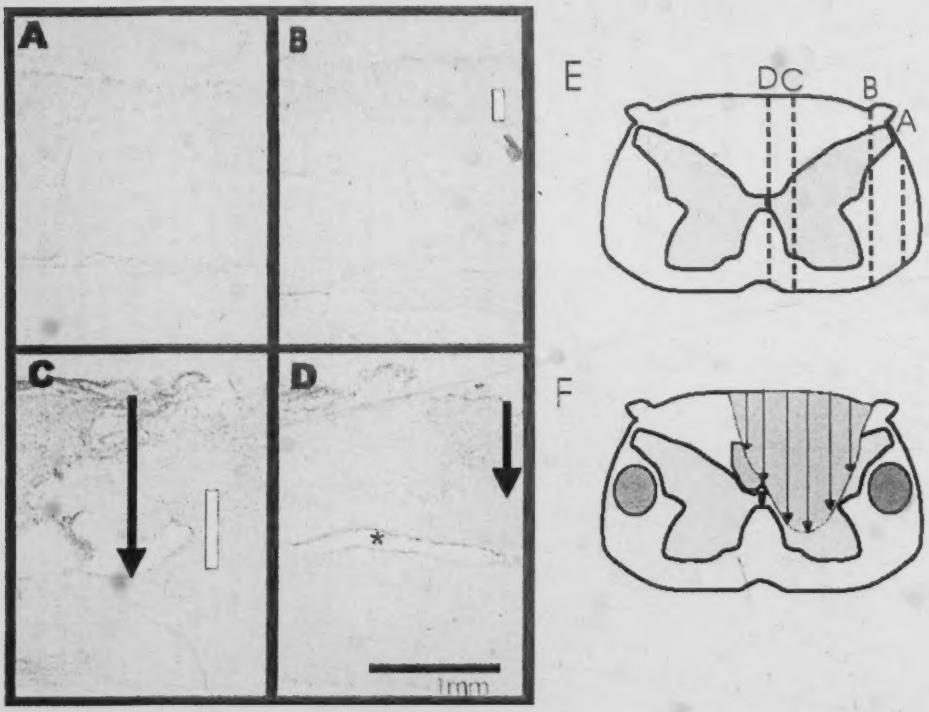

Figure 2. Spinal Cord Histology

Spinal cords were cut parasagittally on a cryostat (25μm) and stained with cresyl violet or silver stain for viewing under a light microscope. **Figure 2A-D** displays 4 parasagittal sections at different mediolateral locations of a single spinal cord. A section composed entirely of lateral white matter is displayed in (**A**), while the image in (**B**) is slightly more medial and a thin band of gray matter (indicated by the white bar) appears near the dorsal (top) aspect of the cord. In (**C**), a broader band of gray matter is located in the middle of the cord (dorsoventrally) and a lesion is apparent on the dorsal aspect, its dorsoventral extent depicted by the black arrow. A fluid-filled cavity is present on the left side in (**C**). The lesion is smaller in (**D**), also indicated by the descending arrow, and the central canal (*) is apparent in the middle of the cord, with very thin bands of gray matter appearing dorsal and ventral to the canal. The labels *A-D* and corresponding dashed lines in **Figure 2E** represent the estimated locations of sections A-D on a 2-dimensional horizontal representation of a spinal cord. The arrows on **Figure 2F** represent the reconstruction of the lesion as it proceeds laterally, slide by slide with the entire lesion estimate noted by the dark, shaded area. The circular shaded region underneath the dorsal horns represent the RSTs, while the central shaded regions above the central canal represent the CSTs.

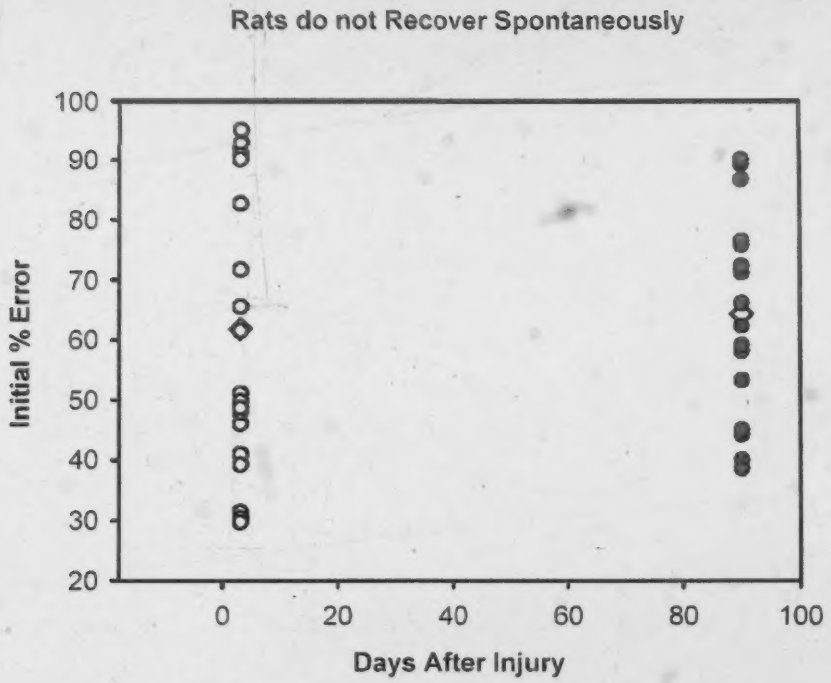

Figure 3. Comparison of Initial Error between Groups
Average initial error and range of error is similar in both groups, with a slightly greater range in the immediate group. The individual values (small circles) and group averages (large diamonds) of error rate on the first day of training are displayed for animals trained immediately (open circles, day 3, n = 21) and after a 3-month delay (solid circles, day 90, n = 16). No significant difference is observed between the 2 groups (two-tailed Student's t-test, p = 0.72). The variability within groups reflects the variability of the lesion size.

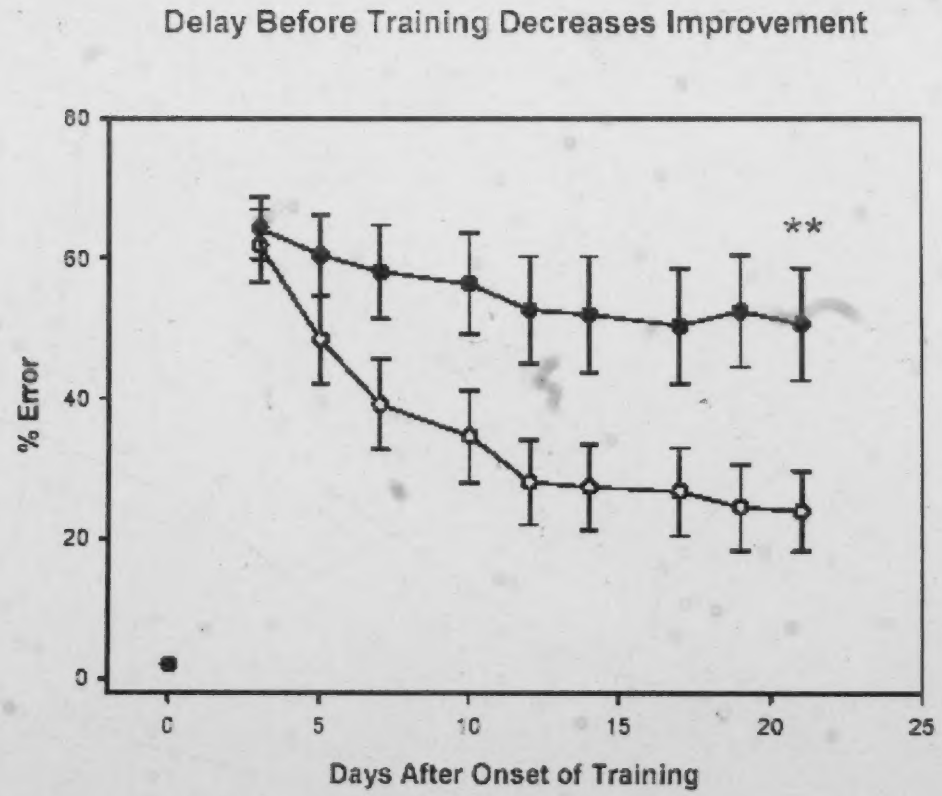

Figure 4. Comparison of Error throughout Training in both Groups
Rats trained immediately after spinal cord injury (following a 3-day recovery period, open circles) demonstrated greater improvement over the 3-week training period than do rats whose training is delayed by 3 months following injury (solid circles). Error is calculated as the number of missteps per step taken x 100%. A student's t-test was used to verify significance of difference (**$p<0.01$). Error bars represent standard error of the mean (SEM). Day 0 is pre-training, pre-lesion, where both groups had an average error rate of 2%; SEM for this point is 0.12%.

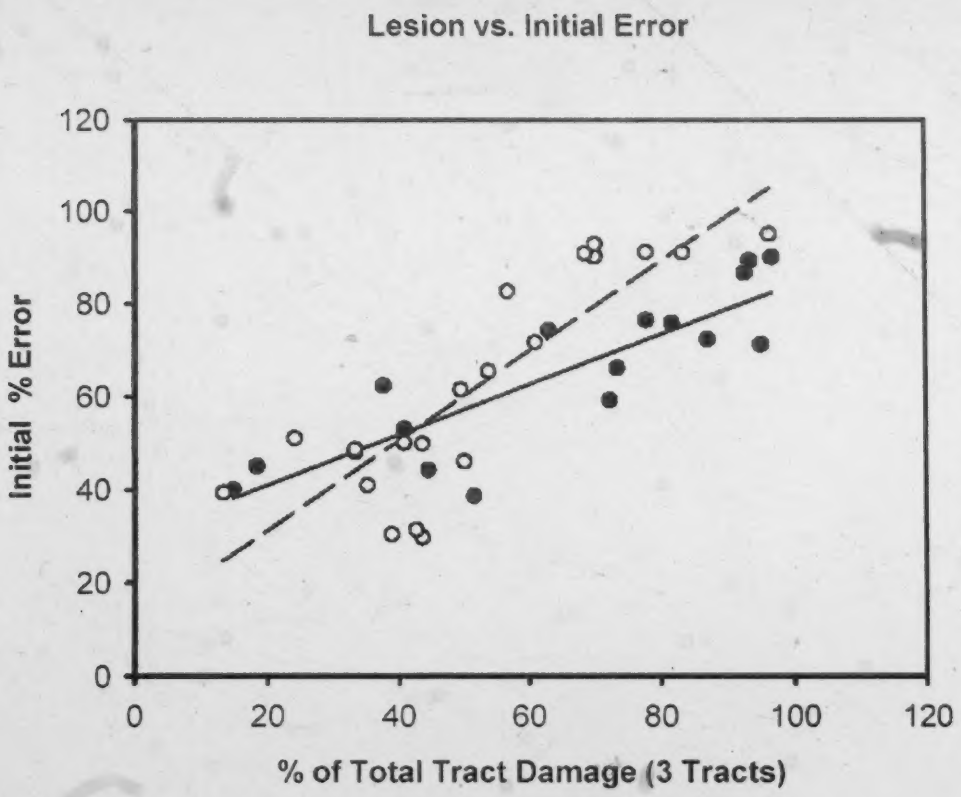

Figure 5. Lesion vs. initial error for total group
Initial error on first day of training plotted against percentage of total tract damage for the ipsilateral CST and RST, and the contralateral CST. Linear regression was applied, and the slope for the immediate group (0.97) was higher than that of the delay group (0.54) due to the immediate group having a greater error for lesions causing > 65% tract damage. Open circles represent immediate, closed represent delay.

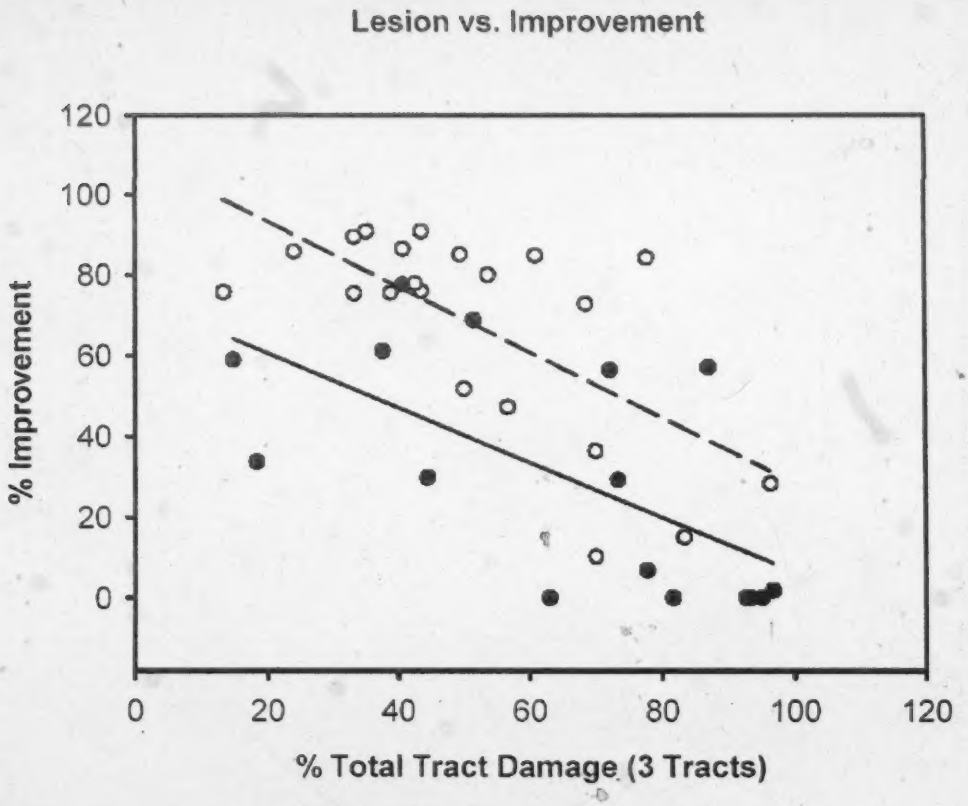

Figure 6. Lesion vs. improvement for total group
Improvement in error over training period ([initial error – final error]/initial error x 100%) plotted against percentage of total tract damage for the ipsilateral CST and RST, and the contralateral CST. Linear regression was applied, and while the slopes of the lines are similar (-0.68 for delay and -0.81 for immediate), the offset (y-intercept) is higher for the immediate group (109.8%) than the delay group (74.3%), indicating greater improvement for similar levels of tract damage. Open circles represent immediate, closed represent delay.

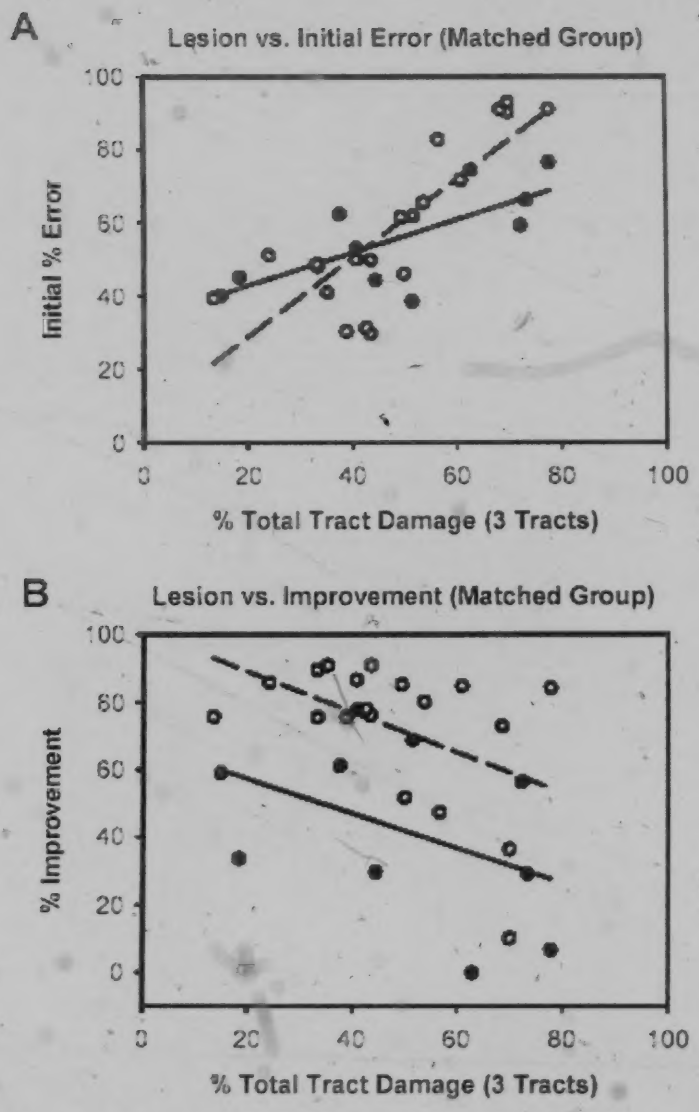

Figure 7. Lesion vs. initial error and improvement for matched group
A) Initial error on first day of training plotted against percentage of total tract damage for the ipsilateral CST and RST, and the contralateral CST, for the matched group of animals. The slope of the regression line remains greater for the immediate group than the delayed group (immediate: 1.07, delay: 0.45).
B) Improvement in error over training period ([initial error – final error]/initial error) plotted against percentage of total tract damage for the ipsilateral CST and RST, and the contralateral CST, for the matched group of animals. Improvement remains greater in the immediate group. The y-intercept values are higher for the immediate group (101.0%) than the delay group (67.5%), but the slope values are similar (delay: -0.51, immediate: -0.60). Open circles represent immediate, closed represent delay.

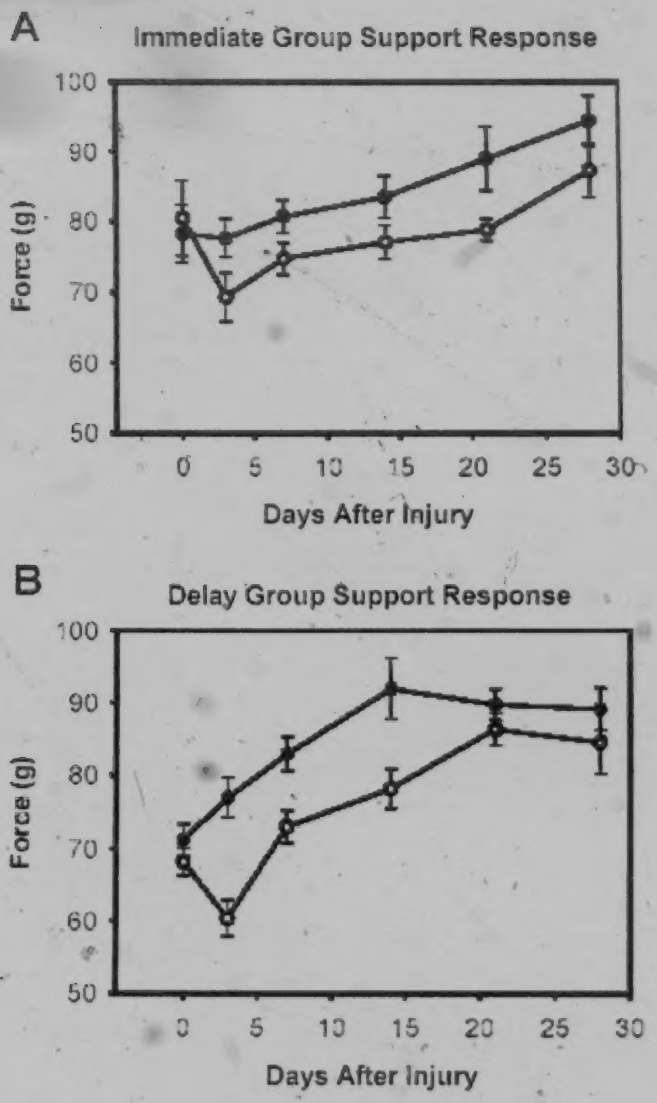

Figure 8. Ground support response in immediate (A) and delay (B) trained animals
A) Ipsilateral ground support response in limbs ipsilateral (open circles) and contralateral to the lesion (closed circles) for animals trained immediately decreased soon after injury, then recovers to near control-limb values (day 28), values given in text. The gradual increase in the support response is partially attributed to weight gain. Error bars represent standard error of the mean.
B) Ipsilateral ground support response for the delay group decreased, then recovered, in a similar manner to that of the immediate group, and although the contralateral response increased more rapidly, the two legs displayed similar responses by day 21.

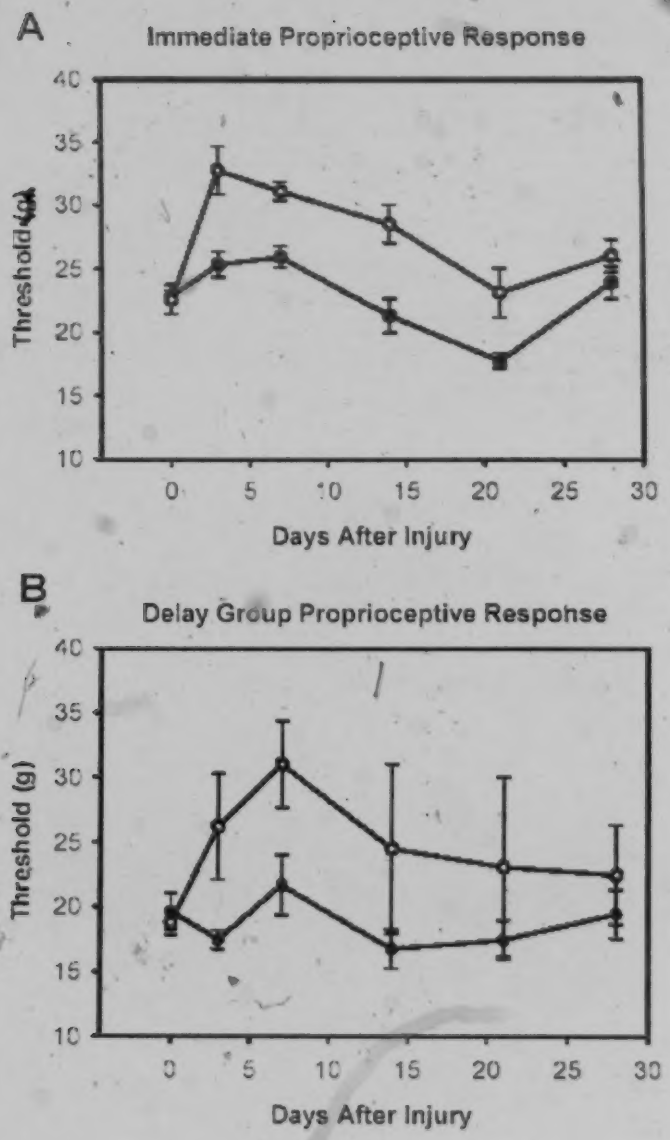

Figure 9. Proprioceptive placing response in immediate (A) and delay (B) trained animals

Ipsilateral proprioceptive threshold was elevated soon after injury in both the immediate **(A)** and delay **(B)** groups, but sensitivity was restored within a 3 weeks. No substantial changes in the contralateral reflex occurred. The profile was fairly similar for the immediate and delayed groups, with maximum increases of 45% (immediate, day 3) and 65% (proprioceptive, day 7), respectively, of day 0 values. Error bars represent standard error of the mean. Open circles represent immediate, closed represent delay.

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<Chapter 3>

Development of Electrophysiological Methods in the Awake Animal to Assess Corticospinal Tract Plasticity

3.1 Introduction

In preparation for further studies, we began preliminary experiments to test the feasibility of conducting long-term electrophysiology studies on animals being trained after spinal cord injury. The major concerns for these pilot studies were to determine if waking animals could tolerate successive electrical stimulation of the motor cortex without undue stress or discomfort; to establish whether motor-evoked potentials (MEPs) can be consistently obtained with our apparatus, with and without spinal cord injury; and to ensure that the stimulating/recording apparatus would remain viable *in vivo* for several weeks (the length of the training period). This chapter describes our preliminary work in this area and outlines studies planned for the future.

Rats that sustain injury to the dorsolateral quadrant of the spinal cord display impairments in their ability to step accurately on a horizontal ladder. If the lesion is not too extensive, daily training can yield dramatic improvements in accurate stepping (Chapter 2). We observed that some correlation existed between responsiveness to training and sparing in any of the ipsilateral or contralateral CSTs or the ipsilateral RST. If all 3 pathways were abolished, virtually no improvements were seen with training,

suggesting that training-mediated recovery depends upon the integrity of at least one of these tracts.

While this provides some anatomical basis for the systems mediating functional recovery due to training, it does not provide a physiological basis. To further elucidate cellular and system mechanisms responsible for behavioral changes, we must identify changes in the cells and motor pathways themselves as they operate, either during the behavior of interest or in similar behaviors which allow a greater degree of experimental control. Electrophysiological studies can be performed to this effect, by combining selective stimulation of centers governing motor control with recording of the activity of the muscles responsible for the actual behaviors.

Spared CST fibers are likely to be involved in the training-related improvements. Thus, improvements in performance may correlate to increases in cortically evoked potentials mediated via the CST. This can be achieved by monitoring the activity in the tibialis anterior, a muscle important for dorsiflexion and accurate foot placement, evoked by electrical stimulation of the motor cortex. With this in mind, we started a preliminary project to ascertain whether EMG responses could be consistently evoked using screws implanted over the motor cortex in an awake animal. We also wanted to see what muscles would be activated by this type of stimulation, if the animal can tolerate the stimulus, and if the implants remain functional for more than a month, i.e., the duration of the training period.

Specifically, electrical stimulation of the hindlimb area of the motor cortex evokes activity in the muscles of the leg, which can be recorded by electromyography (EMG). By recording such evoked responses before injury, immediately after injury, during

training and functional recovery, and for extended periods after injury with no training, we can examine whether the same stimulus will produce different effects in response to the lesion or the training program. A number of options exist with regard to stimulation and recording parameters. Because the majority of the CST decussates in the midbrain, unilateral stimulation to the motor cortex will activate the muscles of the contralateral hindlimb. The leg on the injured side is of primary interest, but this may be compared to evoked potentials in the unaffected leg, to determine if changes are specific to the affected limb.

3.2 Methods

Two Sprague Dawley rats (224g and 240g) were used in this preliminary study with ethics approval obtained from the Health Sciences Animal Welfare and Policies Committee at the University of Alberta. Implantation of cortical and EMG hardware was done under 3% isoflurane anesthesia and aseptic conditions.

3.2.1 Implantation of Cortical Stimulating Screws and EMG electrodes:

Skin and muscles overlying the parietal bone were resected free. A fine drill bit was used to bore holes in the skull, and two screws were placed in the skull at a depth of approximately 1.5mm so as to rest on top of the dura. One screw was placed adjacent to the midline immediately behind bregma, and the other 2mm lateral from the midline and 1mm posterior to bregma, both in the vicinity of the hindlimb area of the motor cortex (as described by (Neafsey et al. 1986). The stimulating screws were pre-soldered to a length of Cooner Wire (AS362) that was run subcutaneously to a machine-pin connector that

was placed in poly-vinyl chloride (PVC) tubing and stored subcutaneously in the animal's back (because we were using 20 channels, we selected this 'backpack' instead of a headpiece, which cannot accommodate so many channels). One drawback is that a fresh wound is created in order to access the device for each recording session. The screw assembly in the skull was covered in quick curing epoxy before closure of the overlying wound.

The EMG electrodes consisted of Cooner wire that was deinsulated by 3mm, frayed, and bent into a hook, and inserted into the muscle with a 22-gauge needle. EMG electrodes were inserted into the medial gastrocnemius (MG), tibialis anterior (TA), vastus lateralis (VL) and posterior biceps semitendinousus (PBST) muscles in Rat# 1 and the TA and MG muscles bilaterally in Rat# 2. The bipolar electrodes were spaced 1cm apart. All wires were run subcutaneously to the machine-pin connector in the animal's back.

3.2.2 Recording of cortically evoked responses in the waking animal.

Before stimulating and recording, the animal was anesthetized with isoflurane (anesthesia was induced with 3% isoflurane and was subsequently varied between 1.5-3% while monitoring the animal's breathing rate) and the machine pin connector located subcutaneously was freed. The animal was placed in a plexi-glass tube to prevent it from disturbing the protruding wires, and allowed to wake up. The screw closest to bregma was used as the cathode. The stimulation parameters were 5 pulses (each lasting 0.25ms, as done by Fouad, personal communication) at a frequency of 330Hz. All EMG channels were amplified 2000 times using custom built preamplifiers having a band-pass of 10 to

10,000Hz. Data were digitized on line using Axon Instrument Digitimer 1302 hardware and Axoscope 8.1 software at a sampling rate of 5000Hz. Although the low band-pass was set to 10,000 Hz, the range in the frequency component of an EMG signal recorded in rats using similar intramuscular electrodes to ours was 100-1,000Hz (Miura et al. 2001). Therefore, the sampling rate of 5,000Hz is more than adequate to record the majority of the signal. Stimulus intensities, ranging from threshold to evoke an EMG response (~4mA) to maximum stimulator output (10mA) were used.

3.3 Results

During sitting, cortical stimulation evoked activity in the contralateral ankle flexor (tibialis anterior: TA), but not the contralateral ankle extensor (medial gastrocnemius: MG), muscle at a latency of 13.5ms (**Figure 1**). A lack of response was also observed in the VL and PBST muscles (not shown). Note that the motor evoked potential (MEP) in the TA muscle was followed by a silent period of almost complete inactivity that lasted for 17ms. The animals tolerated the stimulation quite well and did not respond with vocalization, even though the stimulus intensity was increased to 10mA. In this animal the threshold to evoke a MEP response was around 4.5mA and the peak-to-peak amplitude of the averaged MEP (n = 7 sweeps) increased fairly monotonically as a function of stimulation amplitude (**Figure 2**). Correspondingly, the latency of the MEP decreased as the stimulus amplitude increased; 15.4 ms latency at a stimulus intensity of 5.5mA versus 13.2 ms latency at a stimulation intensity of 10mA, suggesting that as the stimulation intensity increased, and so did recruitment of faster conducting CST axons and/or motoneurons.

In a second animal we tested to see if the supradural electrodes could still produce MEPs in the TA at least 4 weeks after implantation. **Figure 3** (top trace) shows the MEP responses recorded in an animal that was implanted with cortical (left motor cortex) stimulating and EMG electrodes one month earlier. Similar to the first rat, a marked MEP response was only evoked in the contralateral (right) TA muscle at a latency of around 14 ms (distance between first and third dotted line, **Figure 3)**. In some trials, a small response was observed in the MG muscle but only when there was substantial background EMG activity (not shown). After this, a unilateral dorsal quadrant lesion was performed on the left side of the spinal cord, i.e., contralateral to the leg in which we were recording the MEP responses, and the animal was tested again after a one-week recovery period. Following spinal injury a MEP response could still be evoked in the TA muscle contralateral to the injury (bottom trace, **Figure 3)**, indicating that our lesion was restricted mainly to the ipsilateral CST. Note that after the spinal injury, the latency of the MEP decreased to around 10ms (distance between first and second dotted line), even when using the same stimulation intensity of 9mA.

3.4 Conclusions

The stimulation protocol was well-tolerated by both of the animals studied, and the stimulus conditions (threshold, responses evoked at same stimulus intensity) were fairly stable over time (without spinal cord injury). We were able to specifically evoke responses in the contralateral tibialis anterior (ankle flexor), without corresponding potentials occurring in the medial gastrocnemius (ankle extensor). Three weeks later, we were still able to evoke such responses, indicating that the stimulating/recording

apparatus remains viable through the time course of our training protocol. We then performed a lesion on the contralateral dorsal quadrant (sparing the stimulated CST), and demonstrated that we could still evoke activity in the tibialis anterior. Thus, it is feasible to study spared pathways following spinal cord injury with this experimental paradigm, provided enough sparing is present.

In some animals being trained and recorded, sparing may exist in the ipsilateral CST (ipsilateral to the lesion, from the contralateral motor cortex). If this tract is involved in functional recovery related to training, then over the period of training there may be some recovery of an evoked response in the injured leg. Conversely, if sparing exists in the contralateral CST (from the motor cortex ipsilateral to the lesion), then this tract may display changes in the responses evoked by ipsilateral motor cortex stimulation. It is possible that plastic changes take place such that the contralateral CST forms functional connections (whether directly, or more likely through interneurons and possibly propriospinal neurons) to the motor neurons of the affected limb. Increases in crossed spinal connections would be indicated by emergence or improved efficacy of responses evoked by stimulation of the ipsilateral CST (Aoki et al. 1986).

Although approximately 95% of the CST decussates and descends via the dorsal column, a small portion extends along the ipsilateral ventral funiculus. While Whishaw and Metz (2002) found no evidence that the ipsilateral CST contributes to the performance of skilled movements of the forelimb, it is possible that it plays a role in accurate stepping, or accurate stepping after injury. To monitor contributions made by the ipsilateral CST, one could perform large lesions that completely, bilaterally sever the dorsal CSTs, leaving only the ipsilateral ventral CST. While animals with such severe

injuries do not recover accurate stepping, it could be determined whether any changes occur in evoked responses via the ventral CST, with or without training. Spared fibers of some sort are necessary for the recording of cortically-evoked potentials. Even if the CST is completely severed, corticorubral or propriospinal connections may allow transmission of activity.

The next step is to implement periodic recording throughout the training period, in order to examine whether improvements in accurate stepping correlate to changes in evoked responses. EMG recordings could be taken from the TA of each leg, in response to cortical stimulus on either side. Changes in strength of evoked response, stimulus threshold, and latency of response will be noted. Also, the cortex can be stimulated during accurate stepping. Due to extensive co-contraction that occurs during accurate stepping (i.e., TA and MG are co-activated as the animal places its foot on the rung, (Karim Fouad – personal communication), cortical stimulation may result in activation of *both* TA and MG during accurate walking compared to at rest.

The recording will be performed before injury, after injury before training begins, and at the end of each week of training. It is difficult to record more frequently with this technique, as the animal must be anesthetized before each session for surgery to retrieve the connectors. Implementation of an external backpack would render frequent recording more feasible, but also exposes the device to the animal, risking damage.

In addition to simply combining electrophysiology with training, additional surgeries can be performed to better identify which tracts are serving which functions. Transection of the ventral CST, the ipsilateral or contralateral CST at the level of the medullary pyramids, or lesion of the red nucleus can be done to explore the effects on

recovery, with cortically-evoked potentials measured concurrently. By re-sectioning the damaged CST, one can observe whether that tract played any role in recovery, and similarly, by cutting the contralateral CST, one can observe whether the improvements in the leg affected initially will remain or not.

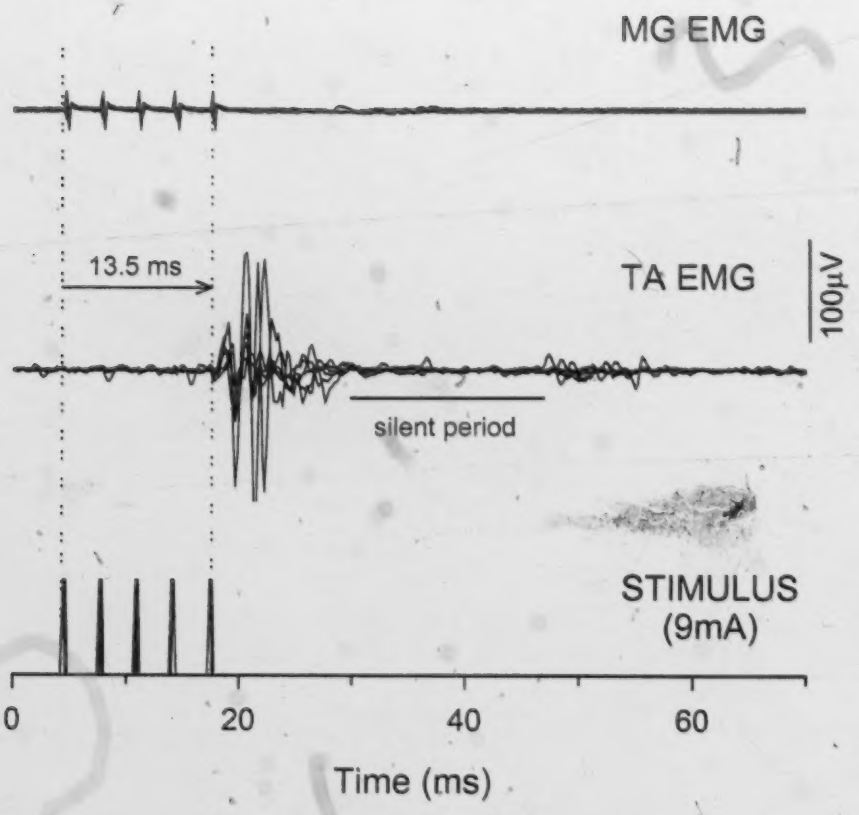

Figure 1 – Stimulation specifically evokes activity in tibialis anterior
A stimulus train evokes a response in the tibialis anterior (TA, middle trace, contralateral to stimulated motor cortex), but not the medial gastrocnemius (MG, top trace, contralateral to stimulated motor cortex). The stimulus train consisted of 5 pulses, 0.25ms wide at 330Hz with an intensity of 9mA. The response in the TA appeared at a latency of 13.5ms, and was followed by a silent period lasting approximately 17ms.

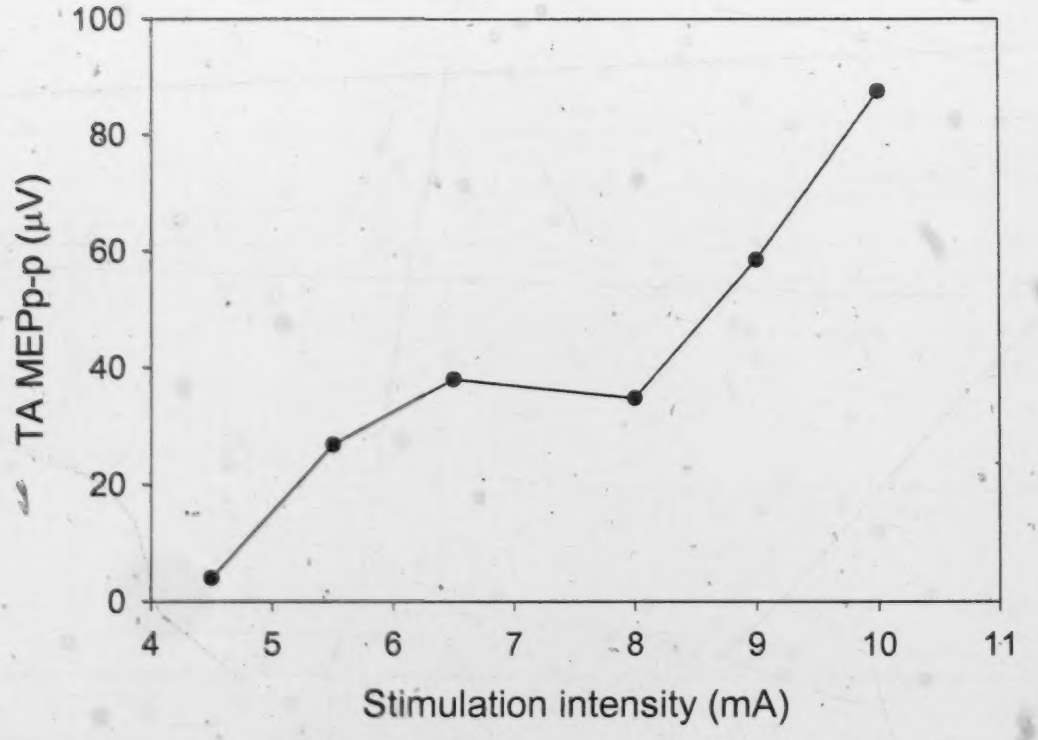

Figure 2 – Stimulus-response curve
The peak-to-peak (p-p) response of the averaged TA MEP is plotted against increasing stimulus amplitudes. Seven sweeps were averaged for each stimulation intensity. A stimulation intensity of 4.5mA was identified as motor threshold. The TA MEPp-p increased linearly with stimulation intensity. Some variability occurred due to changes in background EMG activity.

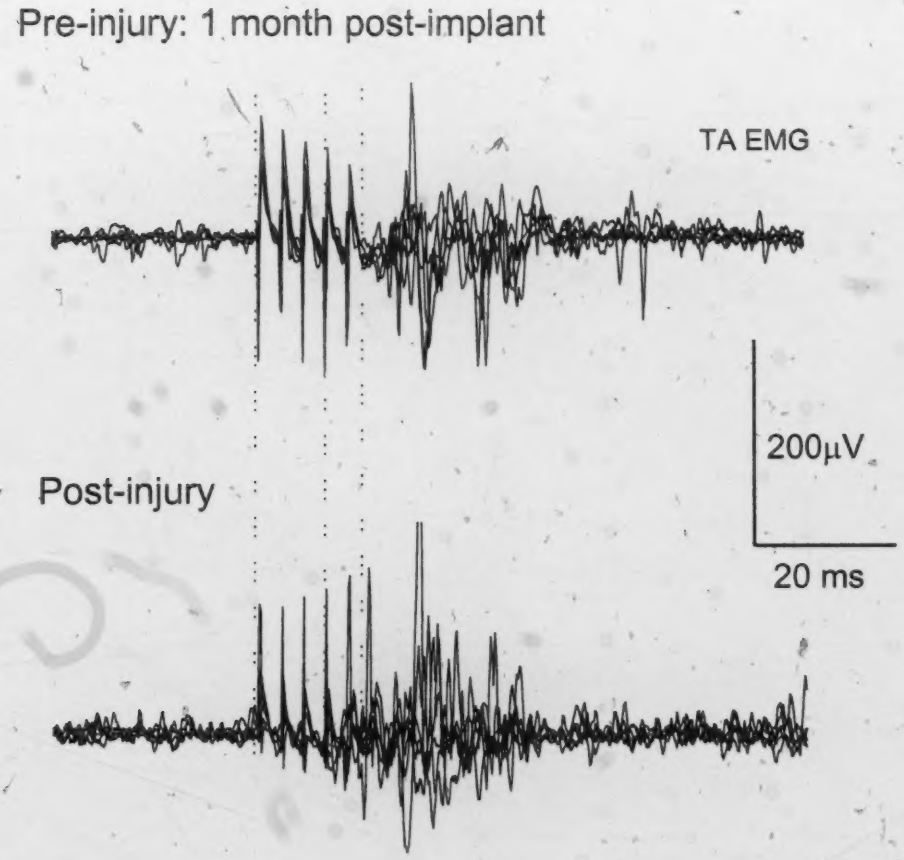

Figure 3 – Evoked response before and after spinal injury
The rat was implanted with stimulating and recording electrodes, which were left in place for a month. One month after implant, evoked potentials were recorded from the contralateral TA at a latency of 14ms (top trace). Following this, a dorsal quadrant lesion was performed on the spinal cord (ipsilateral to the stimulating electrode, aimed so as to not disrupt the evoked response). Responses were successfully evoked again a week after injury, at which time the latency was 10ms (at the same stimulus intensity as before, 9mA, bottom trace).

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<Chapter 4>

General Conclusion

We have demonstrated that daily motor training after spinal cord injury can facilitate improvements in motor skills compromised by the injury, and that delaying the onset of the training has a negative impact upon such recovery. However, a number of questions remain regarding both the nature of the training-induced improvement and the mechanisms underlying the change in responsiveness following the delay period. This section will review some of our results and interpretations in detail, and outline some ideas guiding future study in this field.

In the **General Introduction**, we discussed in detail how motor training can contribute to synaptic plasticity and remodeling of networks responsible for motor functions. Here we will present evidence for changes in candidate pathways, and explore how future studies can be performed in order to determine which descending pathways contribute to training-induced plasticity and functional recovery after spinal cord injury.

The main pathways that are candidates for important contribution in functional recovery with training are the ipsilateral and contralateral dorsal CST, the ventral CST, the ipsilateral RST, and descending propriospinal fibers. Other pathways that may be involved are the contralateral RST, the vestibulospinal tract, and the reticulospinal tract.

The time course of the improvement suggests that unmasking of latent pathways is the main mechanism underlying the relatively quick recovery, as other phenomena such as sprouting of neurites and axonal regeneration take significantly longer (on the order of weeks to months) to occur than the rapid appearance of improvement which is

prominent within the first week of training. This also suggests that neural pathways not normally involved in the control of accurate stepping are far less likely to be contributing to recovery, as presumably those systems are not already in place and thus, would not have time to develop.

4.2 Spared CST/RST Fibers

The main evidence pointing to the contribution of spared CST and RST fibers is the correlation between extent of fiber damage to the initial deficit, and the correlation between sparing of these fibers and eventual improvement. If the damage to all 3 fiber tracts (iCST, cCST and RST) was too extensive, we observed little improvement with training. Conversely, animals with sufficient sparing of these tracts and immediate training exhibited a near complete recovery in ability to walk on the ladder accurately.

Further evidence comes from numerous studies that have demonstrated the roles of the CST and RST in accurate stepping and other skilled limb movements, discussed at length in the **General Introduction** section. Damage to these tracts produces functional deficits, and training can induce rapid improvements in function. The simplest explanation is that training is producing reorganization of the remaining fibers that were already mediating accurate stepping. It has been demonstrated that long-lasting, task-specific functional reorganization of the motor cortex can occur within minutes (Bonato et al., 2002), and that training can promote synaptogenesis, structural plasticity that correlates with functional plasticity (Kleim et al., 2002).

While the existing CST and RST connections represent the most likely basis for functional recovery in our model (especially considering the fast time course of

improvement), a number of other pathways exist that could conceivably transmit information from the motor cortex, where sensory information is processed and a motor plan developed, to the motor neurons of the spinal cord, which directly activate the muscles of locomotion.

4.3 Accessory Pathways

It is possible that the contralateral RST is involved in training-associated recovery of accurate stepping. However, it is unlikely to be a major contributing factor for several reasons. Our histological analysis indicates that the contralateral RST was not damaged in any of the animals studied, yet the severity of initial errors (up to >90%) indicates that despite the full integrity of the RST, little accurate stepping function remains. Furthermore, improvement in accurate stepping correlated to some extent with the sparing of the dorsal CST (ipsilateral and contralateral) and the ipsilateral RST, and when none of these tracts were spared, very little improvement was observed in those animals. Finally, although under appropriate circumstances (in developing animals, or when appropriate growth-inducing and anti-growth-inhibitory factors are applied) the RST can form neurite sprouts that cross the midline following injury, innervating motor systems on the contralateral side of the cord, there is little evidence that these connections exist prior to injury. Once again, it is extremely unlikely that such sprouts could form and generate functionally appropriate synapses quickly enough to mediate recovery within a few days of the commencement of training. The same arguments hold for ruling out the reticulospinal and vestibulospinal tracts as major contributors – no correlation exists between their anatomical integrity and the initial deficits or improvement in accurate

stepping. Moreover, there is very little mention of these tracts playing any role in accurate stepping or related tasks in the literature, so a rapid takeover of the function by these tracts seems unlikely, but remains possible.

4.4 Propriospinal Neurons

There is a complex and extensive network of propriospinal neurons in the rat spinal cord, with reciprocal inhibitory/excitatory connections, including redundant connections (at least in the neonate) that cross the midline of the spinal cord (Cowley and Schmidt, 1997). Retrograde labeling studies have shown that propriospinal neurons project long distances in the rat spinal cord, with cell bodies in the first two cervical segments projecting into ventral horn of the lower lumbar and sacral segments (Miller et al., 1998), with one study estimating that 33% of all fibers in the sacral spinal cord are propriospinal (Chung and Coggeshall, 1983).

In the cat, it has been demonstrated that the corticospinal tract synapses directly onto long and short propriospinal neurons in the cervical spinal cord (Alstermark and Ohlson, 2000). This suggests that the propriospinal pathways may exist as accessory or even redundant routes for the relay of information down the spinal cord. It is conceivable that these connections do not play a primary role in mediating functions such as accurate stepping, but following removal of the CST and RST, and with the appropriate afferent information, the cortico-propriospinal connections may increase their contribution to help restore lost function. Whether this would involve plasticity at the cortical level, or locally at the propriospinal cell bodies, or both, is uncertain.

4.5 Corticorubral System

The pathways most likely to contribute to recovery are the ones whose destruction causes the deficit in the first place. Severity of initial error and, to a lesser extent, eventual improvement, correlated with sparing of the ipsilateral RST and CST, and the contralateral CST. Extensive connections between layer V of the motor cortex and red nucleus have been documented anatomically (Giuffrida et al., 1991), and functionally (Ito and Oda, 1994). Following injury to the neonatal rat CST, a large degree of collateral sprouting from axons of the undamaged motor cortex onto the red nucleus occurs (Z'Graggen et al., 2000), and similar results occur in adult animals treated with IN-1 antibody against growth-inhibitory molecules on myelin (Z'Graggen et al., 1998). However, this type of sprouting is far less prevalent in adult animals without experimental interventions. More importantly, only a small amount of sprouting occurred by 2 weeks following injury, while a major increase was observed after 16 weeks, indicating that the majority of corticorubral sprouting occurs too late to have an impact upon the training-induced improvements we have observed on the horizontal ladder.

4.6 Training and Synaptic Remodeling

Motor training produces structural and physiological changes. While it is well-established that following damage to the cerebral cortex (whether ischemic or traumatic), plasticity in the remaining cortex can take over many lost functions (Hallett, 2001). However, the substrates of reorganization are less straightforward in spinal cord injury, as plasticity may occur at any or all of the following: motor cortex, red nucleus and other

motor nuclei in the brainstem, aspects of the spinal cord rostral to the lesion (propriospinal neurons and interneurons), and aspects of the spinal cord caudal to the lesion (propriospinal neurons, interneurons and the motoneurons themselves). Ultimately, improvements in the strength of connections between the motor cortex and the motor neuron effectors allow improvements in accuracy of precision movements.

There are a number of ways to study the changes in these systems. In **Chapter 3**, we discussed evoking responses in the muscles via cortical stimulation. By observing alterations in connection patterns, such as strength and latency of response, certain assumptions can be made regarding the nature of the connections (i.e., conduction velocity, number of synapses). Anterograde labels can be applied to the motor cortex, allowing visualization of changes in patterns of axonal termination (Kuchler et al., 2002). Changes in synaptic density can be examined at all levels (cortex, red nucleus, propriospinal neurons, interneurons, motor neurons), as well as changes in expression of different neurotransmitters, especially the inhibitory transmitters GABA and glycine (Tillakaratne et al., 2002). Alterations in GABA have previously been shown to contribute to unmasking and fast expansion of cortical representation of movements (Ziemann et al., 2001). Modifications in receptors for neurotransmitters, as well as electrical properties of cellular membranes, can also alter the efficacy of synaptic connections (Wang et al., 2002). By examining the effects of locomotor training on these aspects of spinal cord neurophysiology, it can be determined where (anatomically) and how training brings about functional recovery.

4.7 Effects of a Time Delay before Training

Following injury to the CNS, plastic changes can occur spontaneously. Although the remaining cells may reinnervate denervated areas, the functional integrity is often disrupted. Without task-specific training, the network changes created by spontaneous plasticity may be random (from the point of view of function), and less reorganization occurs. We believe that a window of opportunity exists, during which the system is optimally able to undergo plastic changes that will improve functional performance. If task-specific training is not undertaken during this period, alterations unrelated to the motor task may proceed, reducing the availability of sites of synaptogenesis. Similarly, the presynaptic neurons making new connections may be re-wired in a nonfunctional way, failing to synapse onto appropriate locations. The period of increased plasticity following injury seems to be temporary (Wernig et al., 1998), suggesting that if inappropriate changes are made, they may be difficult to reverse.

Because human locomotor training is less effective if training is delayed for long periods after spinal cord injury, elucidation of the mechanisms is an important first step to improving the functional outcome for these patients. Although notable differences exist between our rat model and human rehabilitative training, functional reorganization may take place in a similar manner, allowing inferences to be drawn about the human system from the animal studies. By gaining a better understanding of how the time-delay impacts training, we may be able to devise therapies to reverse some of the changes that occur during the delay. Such insights could yield increased efficacy to rehabilitative training regimens for people with chronic incomplete spinal cord injury. Perhaps most importantly, with evidence that a time delay has negative consequences upon training,

pressure should increase to get patients to begin rehabilitative therapy as quickly as possible, making functional recovery quicker and more complete.

4.8 Future Directions

In addition to more precise quantification of the lesions with retrograde tracers, we plan to conduct electrophysiological studies, examining any changes in cortically evoked potentials in response to injury, with and without training. Demonstration of functional alterations in descending fibers is necessary to verify that the anatomical changes identified do indeed correspond to the initial deficits and training-related improvements in motor skills. Thus, one might expect increased efficiency of evoked responses in hindlimb muscles due to cortical stimulation after a period of training. Similarly, one might use cortical microstimulation to determine if enlargement of the cortical representation of relevant limb movements (especially flexion of the ankle by the tibialis anterior) occurs. By creating a second lesion, either re-severing tracts damaged in the initial lesion, or by attempting to selectively eliminate previously undamaged pathways, one can observe whether these have any impact on accurate stepping. For example, if you believe that sparing in the ipsilateral motor cortex has contributed the largest amount to functional recovery, this can be tested by transection of this tract at the medullary pyramids (to avoid additional damage to other tracts in the spinal cord, similarly red nucleus ablation can be performed to selectively damage the RST). If no change in performance occurs after this secondary lesion, it is unlikely that the recovery was due to changes in the ipsilateral CST.

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